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Substantial improvements in the prevention, detection and treatment of breast cancer require the addition of new laboratory-trained investigators. The objective of this predoctoral training program is to attract new investigators into the field of breast cancer research and to provide them with an interdisciplinary predoctoral training experience focused on this malignant disorder.

The Program comprises a graduate faculty of 33, who are members of 12 departments at the University of Pittsburgh and are interested in breast cancer and graduate education. An infrastructure has been developed to allow graduate students entering any of the 7 Institutional Ph.D. granting programs to be selected for membership in this Predoctoral Training Program. The Predoctoral Training Program in Breast Cancer Biology and Therapy requires a core of 32 credits of formal course work, including participation in an ethics course, a weekly seminar/research-in-progress series and a newly constructed interdepartmental course on Breast Cancer Biology and Therapy. A minimum of 72 credits with a cumulative grade point average of at least 3.0 must be obtained prior to graduation. Student retention and progress is monitored by the Breast Cancer Training Grant Executive Committee.

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FOREWORD

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____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

9/26/96

PI - Signature Date

D. Table of Contents

E. INTRODUCTION

1. Background

Grants to support training of predoctoral students are usually given to a particular training program in an established scientific discipline or a subdiscipline, rather than for training in a specific disease entity or in a particular model system. Thus, training grants are relatively common in pharmacology, virology, immunology, epidemiology, psychology or biochemistry, regardless of the specific problems various investigators from these disciplines are addressing. What distinguishes these discipline-based predoctoral training programs from our **Training Program in Breast Cancer Biology and Therapy** is our multidisciplinary approach and the focus on a specific and important disease. The overall philosophy of this training proposal is to identify qualified graduate students in existing basic life science departments, to educate them in the problems in breast cancer and to enhance their research capabilities in this field. Our Training Program intends to expand the existing pool of investigators studying breast cancer. Moreover, the program is designed to encourage currently funded investigators to focus on breast cancer as an area of study; this is an important programmatic by-product because it fosters ongoing interdisciplinary research efforts by an array of well-funded investigators.

Breast cancer continues to be a leading cause of death of women in the United States. The magnitude of this disease demands novel approaches to improve our understanding of its etiology as well as methods of detection, prevention and treatment. Yet each year major medical and graduate schools, among them the University of Pittsburgh, continue to successfully graduate hundreds of Ph.D.s impeccably trained in basic principles of endocrinology, molecular genetics, psychology, neuroscience, biochemistry, cell biology, immunology, pharmacology and epidemiology, who have little appreciation for the contemporary problems and advances related to breast cancer. Most of these students have been narrowly trained in the details of their chosen basic science discipline. Very few of these recent Ph.D. graduates pursue postdoctoral training in breast cancer and fewer yet go on to establish successful research laboratories devoted to breast cancer. Part of the problem is the severe shortage of suitable mentors for these students, who could guide them in this direction. Our **Training Program in Breast Cancer Biology and Therapy** is designed to alter this situation. It encourages successful and enthusiastic investigators in various disciplines to address questions related to breast cancer in part by giving them students, whom they can educate while participating in the project; the program emphasizes the multidisciplinary approach to the problem; it educates many young investigators, who would have otherwise worked on other projects, on the importance of breast cancer; it emphasizes the intrinsically interesting biological questions raised by breast cancer and the potential impact and social benefit of working on breast cancer.

F. BODY OF PROPOSAL

1. Technical Objectives

a) Recruit qualified predoctoral students to the **Training Program in Breast Cancer Biology and Therapy.**
b) Educate students in the fundamental principles of breast cancer pathobiology and therapy.
c) Evaluate the progress of the enrolled students.
d) Evaluate the program and seek additional funds and resources.

2. Training Environment

The University of Pittsburgh, founded in 1787, is one of the oldest institutions of higher education in the United States. At present it comprises 16 schools having more than 2,800 faculty and 35,000 students. Of the total student population, 9,940 are currently enrolled in Ph.D. degree programs and 1752 are in professional schools of Medicine, Law or Dentistry. One hundred and fifty students currently are pursuing Ph.D. degrees at the University of Pittsburgh School of Medicine (SOM), 103 Ph.D. graduate students in the Graduate School of Public Health (GSPH) and 17 in the Biopsychology Program of the Department of Psychology in Faculty of Arts and Science (FAS).

The University of Pittsburgh Cancer Institute (UPCI) was established in 1984 to strengthen and expand cancer core and educational resources in the Western Pennsylvania region by developing new, more effective approaches to the prevention, diagnosis and treatment of cancer and by enhancing professional and lay educational programs. This is especially important because the Western PA region has the oldest population of any in the US; this extends to women. Thus, the projected incidence of tumors in the female population is extremely high. In less than 10 years the UPCI has become the major focal point for research and education not only in Western Pennsylvania but also Northern West Virginia and Eastern Ohio; it is now ranked 6th in the United States among recipients for NCI funding with more than $29 million annually; the UPCI has dedicated basic and clinical research facilities totaling more than 250,000 square feet for laboratory studies, 28,000 square feet for outpatient services and over 100 beds for cancer inpatients; over 200 women are treated annually for breast cancer at the UPCI. The UPCI has been responsible for recruiting more than 110 cancer researchers to the Institution, including both the Training Program Director and the Co-Director, and in 1990 the NCI designed the UPCI as a National Comprehensive Cancer Center. Thus, there is a cohort of young and very enthusiastic investigators available for the educational and research mission of the UPCI.

The UPCI is a Vanguard Center for the Women's Health Initiative. In addition, Carnegie Mellon University, which has faculty members in the UPCI and shares

educational programs with the University of Pittsburgh, is physically contiguous with the University of Pittsburgh. Carnegie Mellon has 7,259 students of which 1,164 are enrolled in Ph.D. programs. Thus, within a very small geographical area there is large density of students with a variety of interests and talents. This results in a very dynamic and exciting academic environment, which is conducive for interdisciplinary programs. Indeed a hallmark of the University of Pittsburgh's campus has been the successful development of joint educational program with Carnegie Mellon University, such as the current NSF Fluorescence Center, the NSF/DoD Supercomputer Center, the Biotechnology Center and NIH supported M.D./Ph.D. program (T32-GM08208-06, Joseph M. Furman, PI, 10 positions). As one of the top fifteen recipients of NIH grants, the University of Pittsburgh Medical Center has placed particular emphasis on the importance of external research funds as a vehicle for stimulating high caliber research experience and education. Predoctoral training programs exist in all of the 5 basic medical science departments. The Dean of the SOM provides 22 (one year) predoctoral fellowships annually to these departments. Federal supported predoctoral funds are also available from the NIH Multi-Disciplinary Pulmonary Research Training Grant (2T32-HL07563-07; Robert M. Rogers, PI, 3 predoctoral positions), the NIH Pre- and Postdoctoral Training in Neuroscience (T32-MH18273-12; Michael J. Zigmond, PI, 8 predoctoral positions) and the NIH Predoctoral Training Grant in Pharmacological Sciences (GM08424-03); J. S. Lazo, PI 3 positions). Thus, there is a robust environment for interdisciplinary graduate education in and around the SOM, GSPH and FAS of the University of Pittsburgh.

3. Program Director and Participating Faculty

The **Predoctoral Training Program in Breast Cancer Biology and Therapy** was formally initiated in September 1994 with the awarding of the US Army Training Grant DAMD1-94-J-4039. The Program Director is John S. Lazo, Chair of the Department of Pharmacology and Co-Director of the UPCI Experimental Therapeutics Program, and the Co-Program Director is Olivera J. Finn, Associate Professor of Molecular Genetics and Biochemistry and Director of the UPCI Immunology Program. Dr. Lazo has had more than 25 years of research experience in cancer biology and experimental therapeutics. Much of his early work has been directed at mechanisms of drug action and drug resistance. Most of this research has been tumor type-independent in focus. He has been a member of the Board of Directors of the American Association of Cancer Research and Chair of the 1992 Gordon Research Conference on Chemotherapy of Experimental and Clinical Cancer. He is collaborating with Dr. Olivera Finn to couple antimucin antibodies to DNA cleaving agents and is examining the role of protein phosphatases in breast cancer cells. Dr. Lazo has been a Ph.D. thesis advisor for 2 students, has been a Committee Member for 18 Ph.D. candidates and has trained 21 postdoctoral fellows; he currently is thesis advisor for 2 Ph.D. candidates, both of whom are working on issues related to breast cancer. Two of his previous postdoctoral fellows are now investigating new anticancer agents as clinical pharmacologists at a major pharmaceutical firm (Bristol Myers Beecham) and 1 is designing new diagnostic agents at a biotechnology company. Since 1976 Dr.

Lazo has been intimately involved in both graduate and medical education and since 1979 he has taught a graduate level course almost every year. His basic medical science preparatory book for second year medical students published by Williams and Wilkins (*Review of USMLE Step One*) is among the most popular books of its kind and has entered its fourth edition. Writing and reviewing this book has given the Program Director a broad background in both basic and clinical issues related to malignancies including those associated with the breast. He is also PI of an NIH Predoctoral Training Grant in Pharmacological Sciences. The Co-Director, Dr. Olivera Finn, has been investigating breast cancer biology and immunology since 1985. She has trained 7 Ph.D. students and 10 postdoctoral fellows. Three of her Ph.D. students and 1 postdoctoral fellow have continued their research in breast cancer immunology in their new positions as postdoctoral fellows or assistant professors. She has also been a thesis committee member for 30 Ph.D. candidates. There are currently 5 graduate students in her laboratory, all of whom are doing research in breast cancer. In addition, there are 3 postdoctoral fellows in her laboratory investigating the biology and immunology of breast cancer. In February 1993 Dr. Finn was invited to testify before the President's Cancer Panel, the Special Commission on Breast Cancer, on the future direction of breast cancer and breast cancer vaccines.

The participating faculty members have been drawn from the over 175 members of the Graduate School at the University of Pittsburgh, who are eligible to train students enrolled in a Ph.D. degree granting program. We have selected these 33 faculty members by carefully evaluating them for excellence in the following categories: extramural research support; previous educational experience; research interest in cancer, particularly breast cancer; diversity of research interest and, suitability as a mentor. We have made a special effort to include a significant number of clinically trained investigators (30% of the total faculty have M.D. degrees) to ensure the appropriate exposure of students to clinically relevant issues associated with breast cancer. Participation in this training grant is not viewed as exclusionary and the Training Program Executive Committee throughout the training program funding period will consider new members. For example, this year we added Dr. Victor Vogel, who recently arrived at the University of Pittsburgh, to our Program. Listed below (Table 1) are the members of the faculty, their departmental affiliation and a brief description of their research interests as relate to breast cancer to illustrate the diversity of the faculty members and their interactions.

Table 1. Faculty of the Training Program

Faculty	Department	Major Research Interest
John S. Lazo, Ph.D., Program Director	Pharmacology	Chemotherapy, Drug resistance, Apoptosis
Olivera J. Finn, Ph.D., Program Co-Director	Mol. Genetics & Biochemistry/Surgery	Tumor Immunology, Mucins, Immunogenetics
Edward D. Ball, M.D.	Medicine	Bone marrow transplant., Cell surface markers
Andrew Baum, Ph.D.	Psychiatry/Psychology/Beh. Neuroscience	Behavioral medicine, Stress
Robert A. Branch, M.D.	Medicine	Clinical pharmacology, Drug metabolism
Anthony R. Caggiula, Ph.D.	Psychology	Behavioral immunology, Hormones
David L. Cooper, Ph.D., M.D.	Pathology	Transcription control, Cell matrix, Gene therapy
Andrea Cortese-Hassett, Ph.D.	Pathology	Molecular genetics, Immunology
Billy W. Day, Ph.D.	Environ. & Occupat. Health/Pharm. Sci.	Molec. toxicol., Estrogen, Computational chem.
Albert D. Donnenberg, Ph.D.	Medicine	Bone marrow transplant.
Maryann A. Donovan-Peluso, Ph.D.	Pathology	Molec. genetics, Transcription
Qing-Ping Dou, Ph.D.	Pharmacology	Cell cycle control, Cyclins, Transcription
Roy A. Frye, M.D., Ph.D.	Pathology	Oncogenes, Growth factors, Molec. biology
Joseph C. Glorioso, Ph.D.	Molecular Genetics and Biochemistry	Gene therapy
Ronald H. Goldfarb, Ph.D.	Pathology/Neurosurgery	Metastasis, Invasion, Proteases
Leaf Huang, Ph.D.	Pharmacology/Molec. Genetics & Biochem.	Liposomes, Gene therapy
Candace S. Johnson, Ph.D.	Otolaryngology/ Pharmacology	Exp. therapeutics, Cytokines, Vasculature
Lewis H. Kuller, M.D., Dr.P.H.	Epidemiology	Hormone metabolism, Diet, Endocrinology
Joseph Locker, M.D.	Pathology	Molecular diagnosis, Oncogenes
Michael Lotze, M.D.	Surgery/Molec. Genetics & Biochem.	Gene therapy, Immunotherapy
Susan A. McCarthy, Ph.D.	Surgery/Molec. Genetics & Biochem.	Immunology, T cell function, Apoptosis
Kenneth McCarty, M.D., Ph.D.	Pathology	Steroid receptors, Immunohistochemistry
Edward V. Prochownik, Ph.D., M.D.	Pediatrics/Molec. Genetics & Biochem.	Oncogenes, Early response genes
Paul D. Robbins, Ph.D.	Molecular Genetics and Biochemistry	Tumor suppressor genes, Gene therapy
Guillermo G. Romero	Pharmacology	Signal transduction
Herbert Rosenkranz, Ph.D.	Environmental and Occupational Health	Computational toxin analyses
Russell D. Salter, Ph.D.	Pathology	Immunology, Cell cycle proteasomes
Martin C. Schmidt, Ph.D.	Molecular Genetics and Biochemistry	Transcription factors
Said M. Sebti, Ph.D.	Pharmacology	Signal transduction, Exp. therapeutics, Drug resistance
Jill M. Siegfried, Ph.D.	Pharmacology	Growth factors, Her2/neu oncogene, Tumor vaccines
Victor Vogel, M.D.	Medicine	Biomarkers and Treatment
Theresa Whiteside, Ph.D.	Pathology/Otolaryngology	Immunology, Natural killer cells
Timothy M. Wright, M.D.	Medicine/Molec. Genetics & Biochemistry	Transcriptional regulation, Interferons
Jack C. Yalowich, Ph.D.	Pharmacology	Topoisomerases, Drug resistance, Exp. therapeutics

4. Program

The study of breast cancer biology is a complex area of investigation and further understanding of this problem as well as possible solutions will emerge only through an influx and combined effort of new investigators from many different disciplines of modern biology and science. The overall objective of our **Training Program in Breast Cancer Biology and Therapy** at the University of Pittsburgh is to exploit the well-recognized expertise of selected faculty in Endocrinology, Pharmacology, Psychology, Behavioral Medicine, Medicine, Molecular Genetics, Immunology, Cell Biology and Epidemiology and their specific interests in breast cancer. Recruitment of these investigators from their parent departments into this training program is designed to support their interests in breast cancer and to provide them with an opportunity to recruit and to train young investigators in the basic principles of their discipline using breast cancer as a specific model system.

4.1 Predoctoral Training Pool

There are nine (9) Ph.D. granting programs at the University of Pittsburgh, School of Medicine: Bioengineering (Joint Program between the Schools of Engineering and Medicine), Biochemistry (Granted by the Department of Molecular Genetics and Biochemistry), Microbiology (Granted by the Department of Molecular Genetics and Biochemistry), Pharmacology, Pathology, Cell Biology and Physiology, and Neurobiology. Faculty of the **Training Program in Breast Cancer Biology and Therapy** are members of 5 of these programs. Additional faculty are from one Ph.D. granting program in GSPH, Environmental and Occupational Health, and one Ph.D. granting program from the FAS, Biopsychology. Admission to the training grant program requires a bachelor's degree with a major in chemistry, biology, physics, psychology, microbiology, biology or molecular biology from an accredited college or University, with a minimum grade point average (GPA) of 3.0. In addition, general and advanced subjects of tests of the Graduate Examination Record (GRE) must be taken.

4.2 Program Administrative Structure

The administrative structure of the Training Program uses the resources of existing programs and is chaired by John S. Lazo and co-chaired by Olivera J. Finn. The routine duties such as corresponding with potential applicants, monitoring student progress, ensuring appropriate student records and distribution of information to faculty and students are done by Angie Skender, Administrator of the Graduate Program, Department of Pharmacology. Seminar announcements and journal club schedules have been by Ms. Skender. Ms. Skender meets on a regular basis with the Training Program Executive Committee to evaluate student progress and program needs.

4.3 Student Recruitment and Admission

The recruitment process began in the Spring of 1994 with an effort to identify highly qualified students, who had not yet chosen their research topic or advisor (2nd year students) or who have recently identified a breast cancer related research project (3rd year students). All faculty participating in this training program received a letter informing them of the program and announcements were posted throughout the University. Particular effort will be made to identify and encourage women and individuals from under represented ethnic groups to apply for these fellowship monies.

Applications for admission into the 1994 class of the **Training Program in Breast Cancer Biology and Therapy** were evaluated on June 27, 1994 by the Breast Cancer Training Grant Executive Committee. This Committee comprises the Director and the Co-Director of the program, Drs. Lazo and Finn, and five other faculty members selected for their research interests and diversities. These are: Drs. Kuller, Caggiula, Siegfried, McCarty and Whiteside. Several faculty members had interviewed the candidates and provided information about the applicant, sometimes acting as a formal advocate for the applicant. Each faculty member had one vote and admission was determined based upon total votes awarded each applicant. Applicants were judged based on their undergraduate record, results of GRE scores, performance in first and/or second years of graduate school, faculty comments, and a brief written statement of their research interest as it related to breast cancer. An effort was made to ensure equitable distribution of fellowships between the represented disciplines and areas of research. All awardees were notified within two weeks and no student declined the award. The Executive Committee decided to make a commitment of two years for each student pending successful completion of the first year because this would allow the student security of funding and a more meaningful graduate experience. All students were reviewed in the Spring of 1995 and the Committee renewed their support for a second year.

Applications for admission into the 1996 class of the Training Program in Breast Cancer Biology and Therapy were processed as described for 1994. We received 10 completed applications and each application was discussed in great details (Table 2). The Committee voted not to favor students, whom the Program supported previously. The six finalists are listed below (Table 3).

4.4 Course of Study

A minimum of 32 credits of formal course work and 40 credits of dissertation research are required to earn a Ph.D. in all of the participating departments. The Executive Committee has examined the progress and course grades of the students to ensure they fulfill the requirements of the Program. The students are also required to complete an Ethics course offered by University and to attend the weekly conference on Breast Cancer Biology held by Dr. Victor Vogel, a member of the Executive Committee of this Program. This informal interactive working group was formulated last year and

highlighted only local faculty speakers some of whom are faculty members of this program. In the next year, the seminar series will include outside speakers, who are experts in various aspects of breast cancer biology and therapy. It will also include individuals speaking on behavioral aspects of breast cancer biology and therapy.

The formal course work requirement for most of the departments is similar and is structured around the 4 core courses of (a) Biochemistry, Macromolecules and Metabolism, (b) Cell Structure and Function, c) Molecular Genetics and (d) Signal Transduction. The minimum course work for members of the graduate program in biopsychology are (a) Principles of Behavior, (b) Research Methods, c) Systems Neuroscience and (d) Mammalian Physiology. The second year consists of elective courses. Students supported by the **Training Program in Breast Cancer Biology and Therapy** will be required to take an additional course in breast tumor biology and therapy, which will be offered in the Spring of 1997 and taught by a member of the program faculty. This course will be entitled Breast Cancer Pathobiology and Therapy. An anticipated course outline is seen in Table 4.

Table 2

Applicants	Departments
Marni Brisson	Pharmacology
Albert R. Cunningham	Environmental and Occupational Health
Dana Dellapiazza	Pharmacology
Kirk Dineley	Pharmacology
Cheryl Fattman	Pharmacology
Robert Gealey	Environmental and Occupational Health
Amie McClellan	Biological Sciences
Jennifer Siegert	Molecular Genetics and Biochemistry
James Snyder	Molecular Genetics and Biochemistry
Kristen Veraldi	Molecular Genetics and Biochemistry

Table 3

Student	Year	Mentor	Title of Project
Marni Brisson		Leaf Huang, Ph.D.	Gene therapy in breast cancer
Dana Dellapiazza		John S. Lazo, Ph.D.	Mechanisms responsible for cytoplasmic metallothionein localization in breast cancer cells
Cheryl Fattman		Qing Ping Dou, Ph.D.	Characterization of apoptosis-specific cleavage of the retinoblastoma protein and poly(ADP-ribose) polymerase in breast cancer cells
Amie McClellan		Jeffrey L. Brodsky, Ph.D.	Investigating how heat shock proteins facilitate the translocation of preproteins into the endoplasmic reticulum
Jennifer Siegert		Paul D. Robbins, Ph.D.	Functional interactions between cyclin D1, the retinoblastoma protein (Rb) and components of the basal transcription initiation complex
James Snyder		Olivera J. Finn, Ph.D.	The role of protein kinase C in breast cancer antigen recognition and immunotherapy with T-lymphocytes

Table 4. Course Outline for Breast Cancer, Pathobiology and Therapy

Lecture Block	Block Organizer
Breast Biology (4 sessions)	**K. McCarty**
Topics: Normal Development	K. McCarty
Abnormal Development	K. McCarty
Molecular Genetics and Markers (4 sessions)	**D. Cooper**
Topics: Growth Factors and Signalling	J. Siegfried
Oncogenes	E. Prochownik
Suppressor Genes	D. Cooper
Cytogenetics and Molecular Genetics	J. Locker
Invasion and Metastases (2 sessions)	**R. Goldfarb**
Topics: Extracellular Matrix	D. Cooper
Proteolytic Enzymes	R. Goldfarb
Drug Therapy/Resistance (6 sessions)	**J.S. Lazo**
Topics: Principles of Chemotherapy	J.S. Lazo
Pharmacokinetics and Chemotherapy	J. Yalowich
Cell Cycle Checkpoints	Q. Dou
Apoptosis	J.S. Lazo
Drug Resistance	J. Yalowich
Angiogenesis and Tumor Vasculature	C. Johnson
Immunobiology and Immunotherapy (6 sessions)	**O. Finn**
Topics: Tumor Antigens	O. Finn
T and B Cell Function	S. McCarthy
Natural Killer Cells	T. Whiteside
Vaccines	O. Finn
Adoptive Therapy	M. Lotze
Gene Therapy	M. Lotze
Epidemiology and Prevention (4 sessions)	**L. Kuller**
Topics: Approaches of Risk Factor Assessment	L. Kuller
Data Acquisition and Analyses	K. McCarty
Dietary Control	L. Kuller
Environmental Toxins	B. Day
Behavior (4 sessions)	**A. Caggiula**
Topics: Stress, Immunology and Hormones	A. Caggiula
Psychological Impact of Screening	A. Baum
Promoting Compliance	A. Baum
Behavior and Therapeutic Response	A. Caggiula

4.5 Student Research

A brief summary of the students' progress is given on the following pages.

Ronna Campbell
Interleukin 12 and Breast Cancer

Interleukin 12 is a heterodimeric cytokine, which was purified from an Epstein-Barr virus, transforms lymphoblastoid cell lines based on its ability to stimulate IFN-g production, activates NK cells and acts as a growth factor for T and NK cells. In addition to its immunostimulatory activities, IL-12 has recently been shown to possess an antiangiogenic capacity. IFN-g mediates this antiangiogenic capacity, which is a powerful stimulator of inducible protein-10. IL-12 is produced predominantly by phagocytic cells, including macrophages and polymorphonuclear cells but has recently been produced by epidermoid carcinoma cell lines and normal human keratinocytes. Interleukin 10 inhibits lymphocyte cytokine production, particularly IFN-g from T and NK cells, and inhibits proliferation of T cells indirectly by suppressing monocyte/macrophage production of IL-12. IL-10 is produced by various cell types including keratinocytes, monocytes and B and T cells.

We hypothesized that human breast cancer cell lines, like other epidermoid cell lines, may produce IL-12 and that this may inhibit tumor growth by inducing both nonspecific immunostimulatory activities and angiogenic inhibition. We tested this hypothesis using various human breast cancer cell lines such as BT-20, MDA-NB-435s, ZR-75-30 and others under various culture conditions with and without stimulation. We measured IL-12 production with ELISA and found no IL-12 secretion under every condition tested.

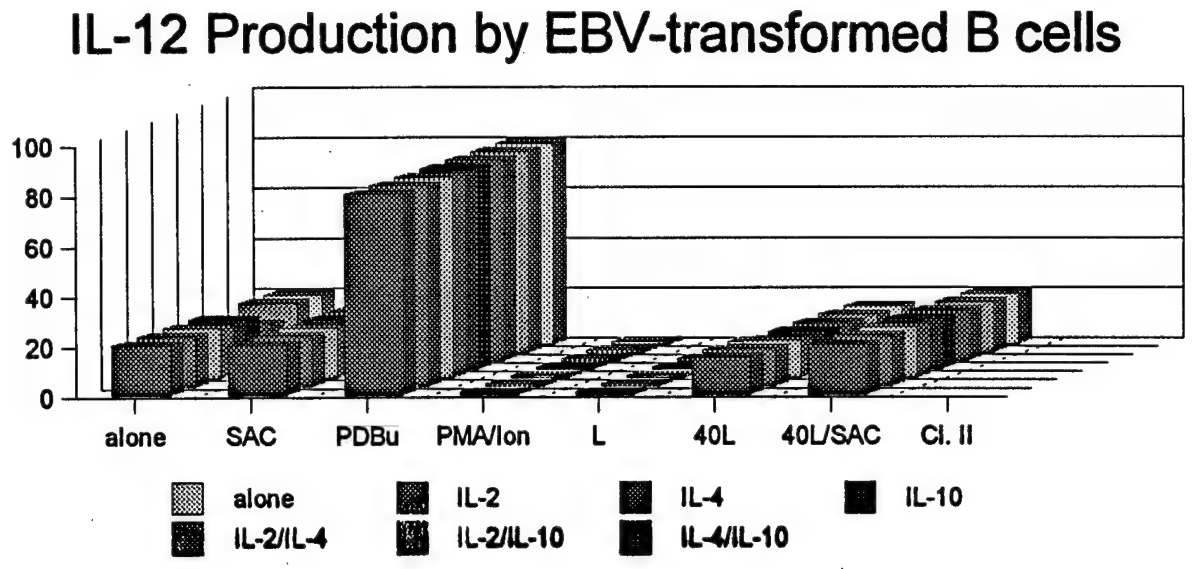
We had previously shown that EBV-transformed cell lines produce variable amounts of IL-12 and that this production is modulated by addition of exogenous IL-10. Due to the absence of IL-12 secretion from breast cancer cell lines we decided to further characterize EBV-transformed cell lines and to look for IL-12 secretion in normal B cells.

To study production of IL-12 we tested multiple mitogens in combination with cytokines, which have been shown to modulate B cell activation. Supernatants collected from purified B cells or cell lines were tested at 72 hours by ELISA for the presence of IL-12. Proliferation assays were carried out in parallel to show stimulatory effects of the mitogens and/or cytokines.

As shown in Figure 1 EBV-transformed B cell line RPMI-8866 can constitutively produce low levels of IL-12, which is dramatically enhanced in the presence of PDBu but not by other mitogens. PMA plus inonmycin completely abrogated IL-12 production

because this combination was toxic to the cells as shown by the lack of proliferation (data not shown). Cytokines appear to have no significant effect on IL-12 production by RPMI-8866 cells.

To study production of IL-12 from normal B cells, we purified splenic and tonsilar B cells. Both populations of B cells failed to produce IL-12 under all conditions tested. To rule out the possibility of autocrine consumption, we tested the cells for intracellular IL-12 by flow cytometry and did not detect any IL-12.



Albert R. Cunningham

Determining Whether a Causative or Modulating Association Exists Between Environmental Estrogen (Xenoestrogen) Exposure and Breast Cancer.

The objective of my research into the etiology of breast cancer is to help determine whether a causative or modulating association exists between environmental estrogen (xenoestrogen) exposure and breast cancer. Since, almost by definition, environmental exposure to xenobiotics occurs on a minute scale (e.g., low chronic doses), a statistically significant relationship between exposure and carcinogenesis is often hard, if not impossible to establish. Furthermore, estrogen induced cancer is believed to occur through a "non-genotoxic" receptor-based mechanism. Although a non-genotoxic route to carcinogenesis is not disputed, the exact mechanism(s) remains elusive. The problem then is (a) to define the mechanism of carcinogenicity of estrogens in humans, (b) to determine if xenoestrogens are included in this archetype and © to determine whether it requires the development of a new mechanism-based risk assessment paradigm. The use

of mechanistic-based information in defining carcinogenic risk to humans has gained acceptance recently.

At the time that I submitted my proposal for this fellowship I was concluding a structure-activity relationship (SAR) study of the carcinogenicity of diethylstilbestrol (DES), a potent human estrogenic carcinogen. The study was conducted with the aid of MULTICASE, a SAR expert-system, and META, an expert-system designed to predict mammalian xenobiotic biotransformation products. Subsequent SAR investigations were conducted into the possible human carcinogenicity of tamoxifen (TMX) and toremifene (TRM), two potent antiestrogenic chemotherapeutics used in the treatment of breast cancer. Although the carcinogenicity of TMX and TRM have not been established in humans, these chemicals are relevant to human carcinogenesis due to their similarities with DES. Also, TMX has been implicated in the induction of rare endometrial cancer in humans as well as the induction of hepatocellular carcinomas and hepatic adenomas in experimental animals.

These studies have yielded insight into the non-genotoxic nature of estrogen-related cancer since neither DES, TMX, TRM nor their metabolites are obvious mutagens. During these studies a 6Å 2-dimensional distance (2D) descriptor biophore associated with carcinogenic estrogens in mice (e.g., diethylstilbestrol and 17b-estradiol) was discovered. The unique nature of the 2D descriptor hypothesis should be noted. A structural feature of chemicals was identified, which is indicative of carcinogens from analyses of a rodent cancer bioassay database. From this SAR model, a testable hypothesis was derived describing a molecular mechanism that may be involved in estrogen-related carcinogenesis. Based upon the presence of this descriptor in carcinogenic estrogens, we suggested that the 2D biophore represented a ligand binding site on an estrogen receptor. This hypothesis was supported by the realization that the biophore was derived from estrogens in the carcinogenicity database and the fact that MULTICASE had been programmed to recognize 2D biophores possessing lipophilic centers as well as moieties capable of hydrogen bonding. These are characteristics associated with ligands that bind to cellular receptors. In contrast to carcinogenic estrogens possessing this biophore, not all estrogenic chemicals contain it, suggesting a dichotomy between carcinogenic and non-carcinogenic estrogens.

A series of phytoestrogens was analyzed for the presence of the 2D biophore. Phytoestrogens have been implicated in lowering the incidence of breast cancer in populations that consume high quantities of these agents (e.g., Asian population with high soy content diets). The results of this study show that all dietary plant derived estrogens tested lack the biophore. This further illustrates the dichotomy between estrogens. Additionally, a series of xenoestrogens was modeled to graphically display the lipophilic nature of each atom in the estrogens. As expected, according to the rules used by MULTICASE in determining 2D biophores, xenoestrogens lacking a lipophilic region para to hydroxyl moieties also lack the 2D biophore. Again, the dichotomy between carcinogenic and non-carcinogenic estrogens was shown.

Currently the focus of my investigations is again SAR analysis of the large carcinogenic potency database (CPDB) of Lois Gold *et al.* The CPDB is a compilation of the carcinogenicity of 1136 chemicals from 4487 experiments. Four rodent subsets have been extracted from this database (i.e., two rodent, a mouse and a rat). A manuscript describing the structural features of the mouse database is ready for submission for publication and will be shortly followed by the rat and rodent reports. The rat and rodent analyses should be concluded quickly since the groundwork for the investigation was laid during the mouse analysis.

In the future I will be assembling several databases describing the estrogenicity of chemicals. With these databases I hope to identify structural features of chemicals indicative of their estrogenicity. This will be a more inclusive investigation than the identification of the previously discussed 6Å biophore. Structural features associated with estrogenicity can then be compared with features related to carcinogenicity as well as other toxicological endpoints. This comparison may reveal if carcinogenicity and estrogenicity are separate phenomena or are intimately related. The mechanism(s) of action of estrogens and xenoestrogens may be further defined. Additionally, structural information about estrogens may help determine whether xenoestrogen exposure is specifically related to the induction of human breast cancer.

The construction and analyses of these databases was a hallmark of my original proposal for this traineeship. Unforeseen studies involving the 2D biophore distracted my efforts to construct these databases. These analyses are still scheduled for completion and should be published as well as contained in my dissertation.

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7. Cunningham A. and Rosenkranz H.S. A study of the carcinogenicity of xenoestrogens: Metabolites of tamoxifen and toremifene. Technical Report NO. CEOHT-95-01 to National Defense Center for Environmental Excellence, 1996. Available on World Wide Web: http://www.pitt.edu/~jyzhang/ctc.

8. Cunningham A. and Rosenkranz H.S. A study of the structural basis of the carcinogenicity of genotoxic and non-genotoxic molecules: Diethylstilbestrol and metabolites: Part II. Technical Report NO. CEOHT-95-01 to National Defense Center for Environmental Excellence, 1996. Available on World Wide Web: http://www.pitt.edu/~jyzhang/ctc.

9. Cunningham A.R., Rosenkranz H.S., Klopman G. and Gold L.S. Structural analyses of cancer causation in mice: The carcinogenic potency database (99% complete).

10. Cunningham A.R., Rosenkranz H.S., Klopman G. and Gold L.S. Structural analyses of cancer causation in rats: The carcinogenic potency database (80% complete).

11. Cunningham A.R., Rosenkranz H.S., Klopman G. and Gold L.S. Structural analyses of cancer causation in rodents: The carcinogenic potency database (50% complete).

Raymond W. Ganster

Mechanisms by which Proteins Regulate Gene Expression through Interactions with TATA-Binding Protein (TBP)

STD1 was cloned independently in high copy number screens for suppressors of mutations in the TATA binding protein (reference 1) and in the SNF1/SNF4 kinase pathway (reference 2). A variety of genetic, two-hybrid, and biochemical interactions suggest that the STD1 protein may couple the glucose starvation signal from the SNF1/SNF4 kinase complex to the general transcription machinery (references 1,2,3). Increased *STD1* gene dosage activates the expression of a number of inducible genes (*CUP1, INO1, SUC2*) under growth conditions that are normally repressive. Double

deletion of *std1*, and a functional sequence homologue *mth1*, can impair the de-repression of *ADH2* and *SUC2* (unpublished data).

A plethora of genes have been identified in primary and secondary genetic screens, which are required for proper regulation of *SUC2* gene transcription by glucose. Among the complexities of loci involved in activation of gene expression by glucose limitation, a number of genes appear to play important roles. For instance, SNF3 is a member of the 12 membrane-spanning hexose transporter superfamily and is thought to be a regulatory glucose sensor. The SNF1-SNF4 kinase complex is required for the proper expression of many genes regulated by glucose. Whereas the SWI/SNF protein complex is a global regulator of transcription that probably functions in the reorganization of nucleosomal chromatin. A variety of biochemical activities have been described for the yeast SWI/SNF protein complex, which could contribute to regulation of gene transcription.

A wide assortment of transcriptional regulators have been isolated in genetic screens, which include repressors (i.e. MIG1, TUP1/SSN6, SKO1, histones and HMG-like proteins) and activators (SW1/SNF complex, MSN2, STD1 (MSN3) and MSN4) of *SCU2* expression. However, scant information exists regarding the *cis*-DNA sequence elements and/or protein interactions through which these factors exert gene-specific transcriptional regulation. For instance, while others have described SWI/SNF complex interactions with nucleosomes in genetic screens and biochemical assays, we have recently shown in a collaboration with the Peterson lab that the SWI/SNF protein complex binds to the minor groove of **naked DNA**. The SWI/SNF DNA binding activity exhibited some sequence specificity and bound with high affinity to synthetic 4-way junction DNA. The synthetic 4-way junction DNA served as an excellent substrate for stimulating the DNA-dependent ATPase activity of the SWI2 subunit. Furthermore, the SWI/SNF complex stimulated positive supercoiling of closed circular plasmid DNA by eukaryotic (but not prokaryotic) topoisomerase, which otherwise creates negative supercoils. We propose that SWI/SNF protein complex may recognize promoter DNA as it exists a "histone" octamer as the topology of this DNA has been shown to resemble a 4-way junction (reference 4). However, the precise mechanism for promoter specific regulation exerted by SWI/SNF complex remains obscure.

Recent experiments implicate STD1 as a Na^+-specific halotolerance factor since overexpression of STD1 dramatically improves growth in the presence of 1M NaCl. The STD1-mediated growth advantage is specific to Na^+ since increased STD1 does not confer a growth advantage in media containing up to 1.5M KC1 or 2M sorbitol. Also, *snf3,* and *snf5* mutant strains are altered in the regulation of gene transcription by glucose and exhibit Na^+-specific growth phenotypes. Growth in the presence of 1M NaCl is also affected by media carbon source or glucose concentration, suggesting a connection between Na^+ and glucose-mediated signalling. The data indicate an involvement of several *SNF* genes that act from the membrane to the nucleus in the gene regulatory response to both glucose and Na^+. Furthermore, genetic interactions between *STD1* and

snf3, snf4, or *snf5* suggest STD1 is an integral component of a glucose-sensitive regulatory pathway that is important in Na^+ ion homeostasis.

Finally, the *HAL1* gene encodes a protein of unknown function that is required for normal growth in the presence of high media Na^+ concentration. Overexpression of HAL1 provides for a Na^+-specific halotolerance, herease *HAL1* knockout strains exhibit a Na^+-specific growth defect. The Na^+-specific growth phenotypes displayed by strains that manipulate *HAL1* gene dosage probably result from *HAL1* gene dose-dependent changes in intracellular potassium ion (K^+). Tight regulation of the intracellular Na /K ratio is regulated by glucose and by *STD1* gene dosage, which may explain the tight connection between glucose regulatory phenomena and Na^+ ion homeostasis (references 5 and 6).

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Dave Krisky

Herpes Simplex Virus Vectors for the Treatment of Brain Cancer

The aim of this project has been to develop Herpes simplex virus type 1 virus (HSV-1) as a gene therapeutic vector for the treatment of breast cancer. Previous progress reports have outlined the production of replication defective HSV-1 vectors which produce

HSV-thymidine kinase (HSV-TK), a potential suicide gene, mIL-4, m-interferon-gamma, or mB7.1. When tested in rat glioma or murine fibrosarcoma tumor models these vectors produce anti-tumor responses which can be classified as minimally significant with respect to survival. The major drawback of these vectors is a high level of virus mediated cytotoxicity which could possibly reduce transgene expression *in vivo* and hence limit effectiveness. These vectors are based upon d120 a KOS strain HSV-1 virus which is deleted for both copies of ICP4 which is a major transactivating protein of the virus (DeLuca *et al.*, 1985). This deletion leads to cessation of production of viral proteins after genes of the immediate early class are expressed and thus after infection only five transcripts (ICP0, ICP22, ICP27, ICP47 and ICP6) are found in abundance. Of these ICP0, ICP22, and ICP27 have been found to produce cytotoxic effects (Johnson *et al.*, 1992; Johnson *et al.*, 1994). In order to reduce cytotoxicity and possibly increase transgene expression additional viral mutants have been produced which contain multiple deletions in the immediate early ICP4, ICP22, and ICP27 genes. In culture these multiply deleted virus show reduced cytotoxicity as measured by ^{3}H thymidine incorporation as well as colony forming efficiency in 9L rat gliosarcoma cells (Fig. 1)(manuscript in preparation). Another disadvantage associated with the use of HSV-1 based vectors is the difficulty of production of recombinant viral vectors. Using standard calcium phosphate transfection procedures for homologous recombination of transgenes into the vector recombination rates of 0-5 % are most common. We have developed a procedure whereby digestion of viral DNA with restriction enzymes which are unique to the HSV-1 genome helps to increase homologous recombination rates when used in conjunction with DNA from mutants which have been engineered to contain the unique enzymes sites (Fig. 2A)(manuscript in preparation). These unique sites have been incorporated into the multiply deleted viral vectors described above thus allowing the production of a new generation of HSV-1 based viral vectors for gene therapy based treatment of human cancers (Fig. 2B).

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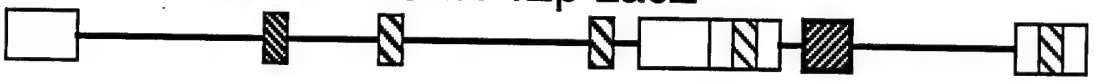
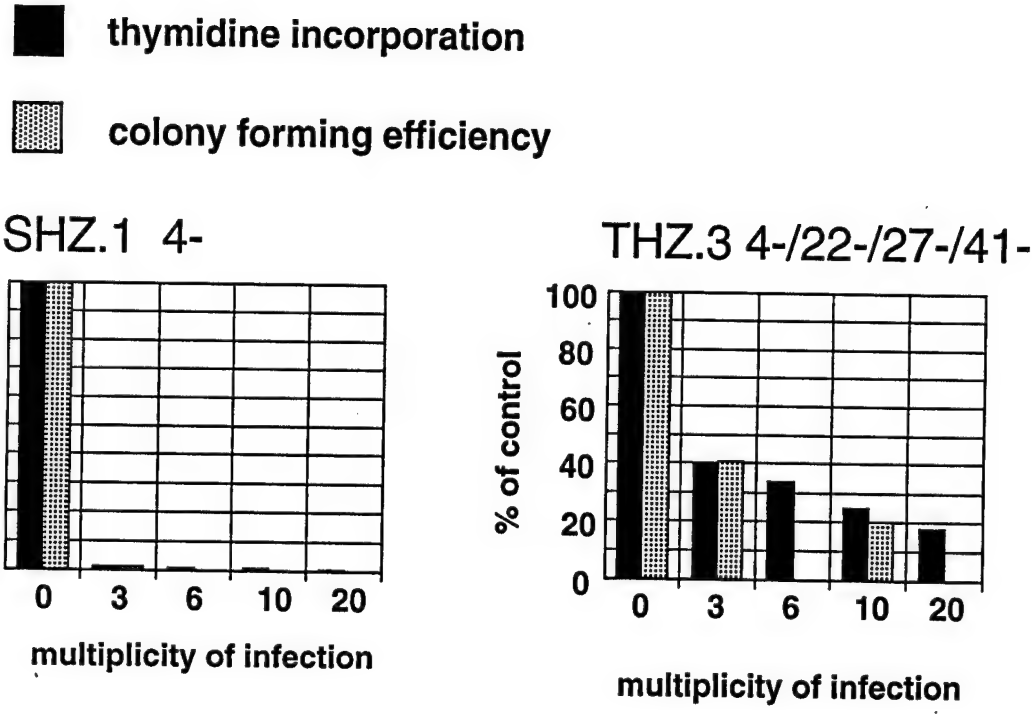
1A

SHZ.1 ICP4-/ UL23- HCMV IEp lacZ

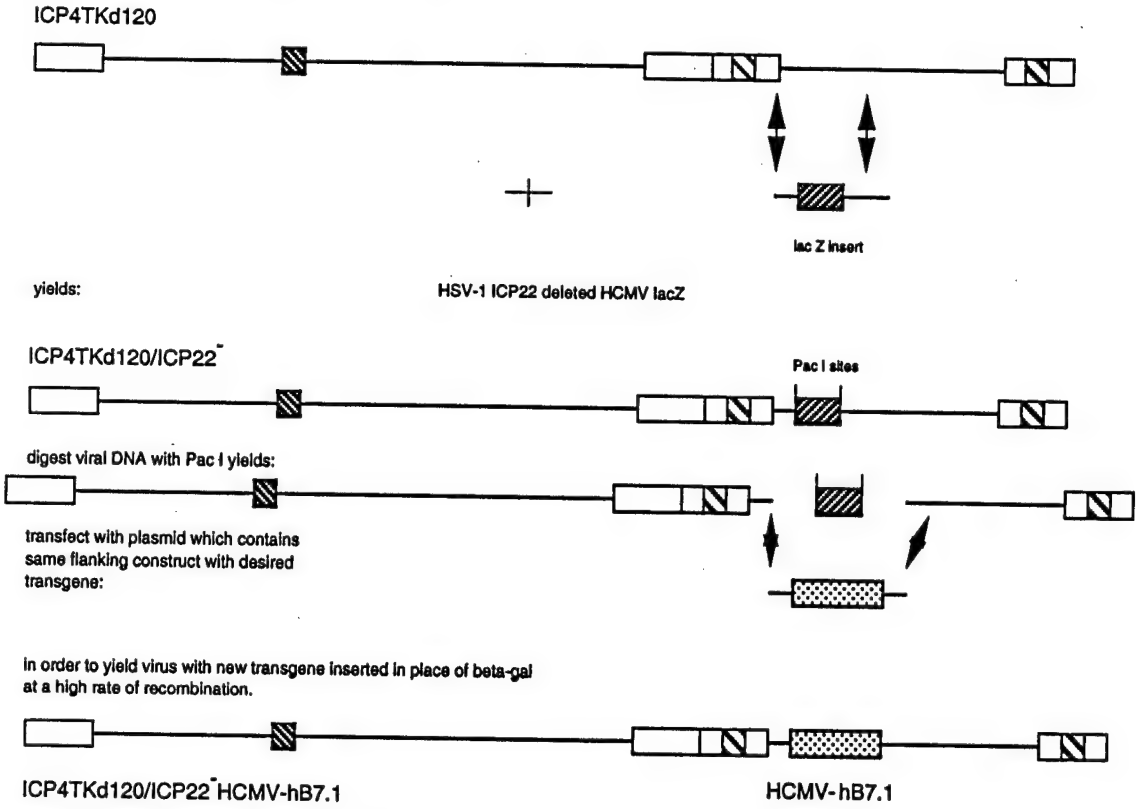
THZ.3 ICP4-/ ICP27-/ UL41-/ ICP4TK UL24-/ ICP22- HCMV IEp LacZ



1B



2A Example of Pac I digestion/recombination repair



This is a schematic of how the virus which contains HCMV-hB7/22⁻ was constructed.

2B Double Pac/ LacZ recombination

viral DNA	insert	%white		%recombination
1. DHZ .1 ICP4-/ICP22- HCMV dp LacZ in ICP22	HCMV-hB7 in ICP22	21/30 45/45	not tested 6/30	20%
2. THZ.1 ICP4-/ICP22-/ICP27- HCMV dp LacZ in ICP22	SCMV-vIL-10 in ICP22	21/24	7/12	~58%
3. SOZ.1 ICP0 dp LacZ in UL41	ICP0-hGM-CSF in UL41	A. 100% B. 100%	7/12	58.3%
4. THZ.1 ICP4-/ICP22-/ICP27- HCMV dp LacZ in ICP22	HCMV-bcl-2 in ICP22	100%	12/24	50%
5. DHZ .2 ICP4-/ICP47- HCMV dp LacZ in ICP47	HCMV-hIL-2 in ICP47	100%	13/20	65%
6. THZ.3 ICP4-/ICP22-/ICP27/UL41- HCMV dp LacZ in ICP22	HCMV-hB7.1 in ICP22	60%	4/6	66%
7. THZ.3 ICP4-/ICP22-/ICP27/UL41- HCMV dp LacZ in ICP22	HCMV-mB7.1 in ICP22	100%	6/20	30%
8. SOZ.1 ICP4-/UL41- ICP0 dp LacZ in UL41	ICP0-mGM-CSF in UL41	80%	3/16	~19%

Without virus flanking regions

1. THZ.1 ICP4-/ICP22-/ICP27- HCMV dp LacZ in ICP22	HCMV-muc1 in ICP22	100%	3/20	15%

Edwina C. Lerner

The Mechanism by which Farnesyltransferase Inhibitors Disrupt Ras/Raf Interaction and Ras signaling in Whole Cells

For the past two years (September 1, 1994 - present), I have been working in Dr. Said Sebti's lab on the mechanism by which farnesyltransferase (FTase) inhibitors disrupt Ras/Raf interaction and Ras signaling in whole cells. Our efforts in this area have resulted in the first direct evidence that non-farnesylated cytosolic Ras can act as a dominant negative inhibitor of Ras signaling and that K-Ras4B, the most abundant mutated Ras in human cancers, may be both farnesylated and geranylgeranylated. These two major findings have a great impact on future research directions in the field of targeting Ras in cancer chemotherapy. This work resulted in two publication is the *Journal of Biological Chemistry* (Appendix). A brief summary of the results is described below.

The Ras oncogene plays a key role in normal cellular proliferation and differentiation and is the most frequently identified oncogene in human cancers. The role of Ras genes in human mammary carcinogenesis remains undefined (1). However, several established human breast cancer cell lines have been shown to contain a mutationally activated *ras* oncogene (2), and 60-70% of hyperplastic lesions and primary breast carcinomas are highly expressive for Ras as compared to normal breast epithelia (1). In addition, while Ras has been identified as one of the most frequently mutated genes found in human cancers, many breast tumors overexpress signal transduction components upstream of Ras (3). For example, many human breast carcinomas overexpress the receptor tyrosine kinases epidermal growth factor (EGFR) and ErbB2. Although Ras is not GTP-locked in these tumors, its activity appears to be upregulated because of the hyperactivity of the tyrosine kinase receptors. Ras is a small guanine nucleotide binding protein that cycles between its inactive GDP-bound state and its active GTP-bound state. Farnesylation and subsequent membrane association of Ras is required for its biological function and its oncogenic activity. Thus, inhibition of Ras membrane association provides a key target for novel approaches to anticancer therapy.

We hypothesized that when GTP-locked Ras accumulates in the cytosol as a consequence of inhibition of membrane association, it would bind tightly to Raf preventing it from localizing to the plasma membrane where it must be present to be activated. We sought to determine the mechanism by which inhibition of Ras processing disrupts Ras downstream signaling by determining what happens to Raf when Ras is removed from the membrane upon treating cells with farnesyltransferase inhibitors. The results from this study are documented in paper A (Appendix). Briefly, cells transformed with a GTP-locked Ras were treated with a highly potent Ras CAAX peptidomimetic FTI-277. Ras was inhibited from going to the plasma membrane and therefore remained in the cytosol where it soaked up Raf, which was also inhibited from going to the membrane where it would be activated. Thus, it appears that GTP-locked Ras when accumulated in the cytosol is

acting as a dominant negative inhibitor resulting in the formation of inactive Ras/Raf complexes, thereby further inhibiting the activation of Ras downstream signaling events, such as MAP kinase (Appendix). These studies provide a potential mechanism by which FTase inhibitors are selective to Ras-transformed tumors over normal cells that do not have a GTP-locked Ras and therefore may not soak up Raf.

The Ras CAAX peptidomimetics are inhibitors that show great selectivity for the enzyme farnesyltransferase over a closely related enzyme geranylgeranyltransferase I *in vitro*, and they selectively block the processing of farnesylated but not geranylgeranylated proteins in whole cells. However, mammalian cells express four types of Ras proteins (H-, N-, K_A-, and K_B-Ras) among which K-Ras4B is the most frequently mutated form of Ras in human cancers. Work by our lab and others has shown the inhibition of H-Ras processing and signaling, but they had not yet shown this with K-Ras4B (Appendix). We were the first to demonstrate the inhibition of K-Ras processing and signaling in whole cells with the highly potent farnesyltransferase inhibitor FTI-277. The inhibition of K-Ras processing, however, occurred at much higher concentrations than needed to inhibit H-Ras processing (Appendix) and were more closely equivalent to concentrations that inhibited the geranylgeranylated protein Rap1A. We, therefore, set out to determine whether a GGTase I-selective inhibitor would disrupt K-Ras4B processing and signaling. Our results demonstrate that oncogenic K-Ras4B processing and constitutive activation of MAP kinase are potently inhibited by a GGTase I-selective inhibitor (GGTI-286 and GGTI-298) but are resistant to one selective for FTase (FTI-277) (Appendix), indicating that K-Ras may be both farnesylated and geranylgeranylated in whole cells. The results presented in this study are critical to the future development of inhibitors of Ras prenylation as potential anticancer agents. This is a key finding since K-Ras4B is the most frequently identified mutated Ras in human cancers and its function has been resistant to FTase inhibitors.

The above results were demonstrated in NIH 3T3 cells transfected by various Ras oncogenes, and should serve as a great model to understand the function of Ras and the mechanism of action of the Ras CAAX peptidomimetics in human tumors. However, the sensitivity of the FTase inhibitors (FTI's) and GGTase I inhibitors (GGTI's) in human tumors on the prenylation of oncogenic Ras proteins in not known, and the picture is more complicated because of the expression of all three Ras proteins (H-, K-, and N-Ras). While the presence of Ras mutations in human breast cancers remains low (~5-20%) in relation to other tumors such as of the pancreas, it is very important to determine whether inhibition of farnesylation and/or geranylgeranylation is sufficient for the inhibition of human tumor growth where Ras is mutated. We have identified a pancreatic tumor line (PANC-1), colon carcinoma cell lines (Colo 357, Colo 205), a bladder carcinoma cell line (T24), and Breast carcinoma cell lines (MDA-MB134, MDA-MB231) which carry a Ras mutation, and we have learned the effects of the FTI's and GGTI's on the inhibition of H-, K-, and N-Ras prenylation, and studies are currently underway to determine the effects of the inhibitors on the growth of these cells in soft agar. We have demonstrated that all three Ras proteins are expressed in the above human tumor cell lines, and that FTI-277 is highly potent at inhibiting H-Ras and N-Ras prenylation, which is consistent with previous data

from the H-and N-Ras transformed NIH 3T3 cells. In addition, we have determined that the inhibition of K-Ras prenylation by FTI-277 occurs only at concentrations that begin to effect geranylgeranylation (>25 uM); and K-Ras prenylation is most inhibited in these tumor cells when both the FTase inhibitor and the GGTase I inhibitor are used, indicating that K-Ras may be both geranylgeranylated and farnesylated in human tumors. Preliminary results indicate that the growth of these cells in soft agar is affected by FTI-277 and to a greater extent GGTI-298, and the co-treatment with both FTI-277 and GGTI-298 totally inhibits the growth of these cells in soft agar. Again, these studies are presently ongoing, and the effects on cell growth will be correlated to the inhibition of Ras prenylation.

A critical question in the field of FTase inhibitors is whether disruption of Ras function would result in inhibition of tumor growth in human cancers where Ras is not mutated, but where its function may be aberrant because of other genetic alterations. This is an extremely important question because if the inhibitors are effective in these cells, such a finding would increase the spectrum of tumors that can benefit from these inhibitors. Therefore, studies are presently underway to determine the effects of the FTI and GGTI compounds in breast tumor cell lines (SKBr-3, BT474, MDA-MB453, MDA-MB468, MDA-MB361), which overexpress the EGF, ErbB2, and ErbB3 receptors, elements that are upstream of Ras but where Ras is integrally involved in these signaling pathways. We have determined that these cell lines express all three Ras Proteins (H-, K-, and N-Ras) and the inhibition of Ras prenylation by the FTI and GGTI compounds is characteristic with previous data listed above with cell lines, which carry an oncogenic Ras. We are currently attempting to correlate the Ras prenylation data with the effects of the inhibitors on the growth of these cells in soft agar. In addition, studies are currently underway to investigate the ability of the inhibitors to block Mitogen Activated Protein (MAP) kinase activation, a downstream signaling molecule of Ras, and to determine if the GGTase I inhibitors effect the tyrosine phosphorylation levels of the EG, ErbB2, and ErbB3 receptors (Dr. Terence McGuire in our lab has demonstrated that GGTI compounds can block PDGF-stimulated tyrosine phosphorylation of the PDGF receptor).

Publications while under the Breast Cancer Predoctoral Training Grant:

A. Lerner, E.C., Qian, Y., Blaskovich, M.A., Fossum, R.D., Vogt, A., Sun, J., Cox, A.D., Der, C.J., Hamilton, A.D. and Sebti, S.M. Ras CAAX Peptidomimetic FTI-277 Selectively Blocks Oncogenic Ras Signaling by Inducing Cytoplasmic Accumulation of Inactive Ras/Raf Complexes. J. Biol. Chem. 270:26802-26806, 1995.

B. Lerner, E.C., Qian, Y., Hamilton, A.D. and Sebti, S.M. Design of a Potent Geranylgeranyltransferase I Inhibitor: Disruption of Oncogenic K-Ras4B Processing and Signaling. J. Biol. Chem. 270: 26770-26773, 1995.

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Diane M. Zeleski

Insulin Receptor Substrate-1 Expression and Function in Human Breast Cancer Cell Lines

Hypothesis

A calculated reduction in insulin receptor substrate-1 protein levels can reverse the tumorigenicity of breast cancer.

Background and Significance

The insulin receptor (IR) and insulin-like growth factor-1 receptor (IGF-1R) are members of the tyrosine kinase growth factor receptor family. The activation of tyrosine kinase receptors plays a key role in both normal and neoplastic cell growth. Numerous studies support the role of signaling through the IGF-1R as very relevant to breast cancer proliferation. Although the epithelial cells under study do not secrete their own IGF-1, in their natural environment they obtain it in a paracrine fashion from nearby stromal cells. They do, however, express the receptor for it, are sensitive to IGF-1, and the IGF-1R has been found to be amplified in 10-15% of primary breast tumors. IR over-expression, on the other hand, is capable of inducing a ligand-dependent transformed phenotype. A sixfold overexpression of structurally and functionally normal IR content has been reported in human breast cancer specimens and many cultured breast cancer cell lines. Additionally, IR content has been found to correlate with other clinical parameters, including tumor size and grade, that reflect increased tumor aggressiveness. Since the receptor numbers have been established to be aberrant, the possibility exists that by short-circuiting the signal transduction pathway just proximal to the receptors, a reversal of the tumorigenicity of these breast cancer cell lines may be realized.

The insulin receptor substrate-1 (IRS-1) protein is a potentially good candidate to target for effective short-circuiting of these signaling systems. Numerous studies have reported that IRS-1 is an important phosphoprotein central to many of insulin's and IGF-1's signaling pathways, and it functions just proximal to both growth factor receptors. Fortunately, the rat, mouse, and human IRS-1 sequences have recently been cloned. The

three protein sequences are highly conserved (>90%), particularly in their potential phosphorylation sites. This high degree of conservation across species supports the notion of the central importance of IRS-1 in both insulin and IGF-1 signaling. IRS-1 contains at least a dozen tyrosine residues and nearly 50 serine/threonine residues as potential phosphorylation sites. Although the *in vivo* phosphorylation status of IRS-1 has not been thoroughly characterized, the enormous number of such sites infers that it is a highly regulated phosphoprotein in downstream modulation of growth factor activation. Immediately following insulin/IGF-1 stimulation, IRS-1 is rapidly phosphorylated on tyrosine residues. Tyrosyl phosphorylated IRS-1 is known to interact with the SH2 domains of several critical signaling proteins. These include: the regulatory 85 kD α subunit of PI3K; the tyrosine phosphatase, Syp; the adaptor protein, Grb-2, essential for p21ras activation; and oncogenic adaptor proteins , such as Nck. An observation of considerable importance is the fact that the functional expression of IRS-1 protein has recently been established as an essential component for the mitogenic response in a CHO cell line. Given the fact that both insulin and IGF-1 stimulate proliferation of breast cancer cells and IRS-1 is a central component of this signaling, the study of the role of IRS-1 in the pathobiology of breast cancer is of considerable interest.

Results: First Year of Support

The candidate is currently doing thesis research in the laboratory of Professor Guillermo Romero in the Department of Pharmacology. Over the first year of support on this grant, preliminary studies focused on IRS-1 knockout experiments utilizing antisense strategies in a murine NIH3T3-derived fibroblast cell line, F442A. Antisense constructs were generated in the established eukaryotic expression vector, pcDNA3 (In Vitrogen), carrying antisense (or sense) versions of the rat IRS-1 cDNA (78%, nt +143 to +2613) or a synthetic 57-mer (nt -9 to +41) targeted at the initiation site. This vector is equipped to produce high-level constitutive transcription from mammalian enhancer/ promoter sequences (CMV). Cationic liposome-mediated transfection (with Lipofectamine, Gibco, BRL) of these constructs was completed in the F442A cell line, and selection and propagation of single clones stably expressing the constructs was achieved. Analysis by SDS-PAGE, Western blot, and densitometry revealed that the antisense constructs exhibited 52% (cDNA) and 27% (57-mer) reduction in IRS-1 protein content relative to the mock transfection control.

These preliminary studies are being extended to several breast cancer cell lines. Three of the initial eight breast cancer cell lines screened are being retained for further studies based upon basal IRS-1 content (W. blot) and estrogen receptor (ER) status, the latter an important indicator of the success of breast cancer therapy. The cell lines to be examined are: (1) MDA-MB-231 (high IRS-1, ER-); (2) T47D (moderate IRS-1, ER+); and (3) SKBR3 (moderate IRS-1, ER to be determined). Since the candidate will be focusing on mitogenic assays, these cell lines are being grown in RPMI 1640 media (+10% FBS,

+1% penicillin/streptomycin) minus phenol red, which has been shown to exhibit estrogen-like effects on estrogen-sensitive cell lines.

Results: Second Year of Support

Based upon the preliminary results obtained in the first year of support (and in consultation with thesis committee members), it was suggested that an inducible promoter expression system might prove superior to the constitutive expression of the antisense sequences obtained from the CMV promoter of pcDNA3. The rationale to support this switch in expression vectors is based on two observations. First, if IRS-1 is indeed essential for the mitogenic response, as reported by three separate laboratories in the literature, then selection with G418 in the first transfection experiments may have selected for only those clones in which IRS-1 expression was not severely compromised. This would then prove to be a self-defeating experimental design. Indeed, the maximal reduction of IRS-1 in the first series of transfections was no more than 52%. Secondly, transient transfection of the breast cancer cell lines with a *lac z*-containing construct (followed by staining with the blue substrate, X-gal) revealed less than 5% transfection efficiency, and this coupled with the fact that IRS-1 has a significantly long protein half-life (~12-20 h) implies long-term selection is essential to delineate any observable changes in downstream signaling caused by the antisense. It is envisioned that a further decrease in IRS-1 expression might be realized via utilization of an inducible expression system.

Therefore, the major objective of the second year of support was to return to cloning the antisense sequences into an inducible expression system. Inducible promoter systems offer the advantages of on/off regulation as well as the achievement of a limited or graded expression. The expression system of choice was the tetracycline (tet) -regulated system originally constructed by M. Gossen and H. Bujard in 1992 (*PNAS* '92, 89:5547) and modified in 1995 by D.G. Schatz, *et al.* (*PNAS* '95, 92: 6522). This system utilizes two separate vectors, one in which to clone in the gene of interest (antisense sequences in this case) and the second which expresses a transactivating factor, termed tTA, which fuses the DNA-binding tetracycline repressor protein with the C-terminal activating domain of virion protein 16 of herpes simplex virus. Both vectors contain a minimal CMV promoter under tet regulation by the presence of a heptamer of the Tn*10*-specified tet-resistance operon of *E. coli* [$(\text{tet o})_7$]. This dual vector system is virtually silent in the presence of 0.1 μg/mL tet (added to cell culture media or the drinking water of mice), but exhibits induction levels of up to five orders of magnitude within 48 h after removal of tetracycline from the system. The modified tet-regulated system is superior to other inducible expression systems in several respects, particularly a decrease in leakiness in the uninduced state, as well as an absence of pleiotropic effects, often problematic in other mammalian inducible expression systems. Despite its recent development, this modified system is already commercially available (Gibco, BRL), and several publications support its successful and superior application in various research settings.

To date, subcloning of three antisense sequences into the new shuttle vector (ptet-splice) has been completed, and transfection of the breast cancer cell lines with the tTA-containing vector is about to commence. The three antisense sequences to be employed are: (1) the same cDNA (78%, nt +143 to +2613) that was employed in the first round; (2) a human-specific (the breast cancer cell lines are of human origin) 54-mer (nt -30 to +18); and (3) a shorter 29-mer (nt -5 to +18) that is conserved among all three recently cloned sequences of IRS-1 (human, mouse and rat). The sense version of the cDNA as well as sense and random versions of the oligomers will be used as negative controls. In addition, mock transfection with the vector backbone alone will also be employed as a control.

In addition to the cloning procedures, other corollary studies were conducted during the second year of support. The results of experiments aimed at determining if IRS-1 is tyrosine phosphorylated, and therefore activated, in the basal state in these breast cancer cell lines (as discussed in last year's progress report) have been dubious. A panel of commercially available monoclonal (and polyclonal) anti-phosphotyrosine antibodies was implemented in a variety of experiments (immunoprecipitation and immunoblotting) to examine this possibility. The conditions were modified in many ways, with the antibodies sometimes used as single reagents while other times used in cocktails, yet the results were repeatedly inconsistent. The answer to this question is unclear, and this may be due to the possibility that these antibodies are only able to recognize a restricted subset of tyrosine phosphorylated proteins based upon the nature of the immunogen used to generate the antibodies. This phase of the study may be resurrected at a future date based upon the availability of more useful antibodies.

As mentioned earlier, the responsiveness of the breast cancer cell lines to IGF-1 is probably even more relevant than to that of insulin in the study of breast cancer. To reiterate, IRS-1 is also central to signaling through the IGF-1 receptor. Therefore, these cells were screened in a preliminary manner and found to be IGF-1 responsive; however, a single dose of IGF-1 was employed in this initial examination, and further experiments will need to be conducted to test the optimum dose of IGF-1 to be utilized in future work.

In conjunction with these experiments, less interesting, but necessary, studies were also undertaken.

Experiments were performed to determine the best cell density for IRS-1 expression (W. blot) in tissue culture plates, revealing cells growing in mid log phase to express the highest levels of IRS-1. Currently, automated sequencing of the new constructs is in progress. In addition, vectors containing reporter genes (luciferase and β-galactosidase) as well as vectors controlling the expression of resistance genes (zeocin, neomycin, hygromycin) to be used for long-term selection of the double-transfectants are being analyzed, purified and amplified. Finally, a new commercially available polyclonal antibody directed against IRS-1 (UBI) has been tested and is now being used to supplement our dwindling stock of anti-IRS-1 antibody that had been a generous gift from

another laboratory. Therefore, a variety of other activities have been ongoing during this period of time that are supportive to the central goals of the project.

Future Work

Although financial support for the candidate under this training grant will end in less than one month, continued studies are planned as this research has developed into a thesis project. The Principal Investigator of this laboratory has applied for continued support of this project through an independent proposal application to the U.S. Army.

The constructs generated will be verified by automated sequencing. The protocol requires sequential transfections, selection and propagation of single clones stably expressing the two independent constructs. The first round of generating single clones should prove valuable, as breast cancer cell lines stably expressing the tTA construct will lend themselves to many other studies outside the realm of this laboratory. Once the second round of single clones are generated which stably express both the tTA as well as the antisense constructs, future studies will examine the mitogenic assays of cell proliferation (3H-thymidine incorporation), phosphatidyl inositol 3-kinase (PI3K) activation, as well as mitogen activated protein kinase (MAPK) activation. All of these assays are established techniques employed in our laboratory. In addition, cellular changes in morphological appearance, serum dependence and growth in semi-solid medium (0.41% agar) will be assessed.

Beyond this stage of the project, studies will move toward analysis of tumor cell growth in nude mice. A recent study has already shown that antibodies directed against the IGF-1 receptor in MDA-MB-231 cells inhibits tumor cell growth in nude mice. Therefore, it is anticipated that the transfected cells containing the antisense constructs from this project will also exhibit less tumorigenic potential than the controls, and may prove to be a more efficacious target than the receptors themselves. The mitogenic response arising from the activation of tyrosine kinase signal transduction pathways plays a pivotal role in the pathobiology of certain breast cancers. Consequently, these studies, in particular the development of the tTA-containing breast cancer cell lines, will allow more effective targeting of gene therapy approaches for the treatment of those breast cancers.

APPENDIX

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A dichotomy in the lipophilicity of natural estrogens/ xenoestrogens and phytoestrogens

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A dichotomy in the lipophilicity of estrogens

Key words: CASE/MULTICASE, cancer, estrogens, lipophilicity

Abbreviation: MMP; Molecular Modeling Pro

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Abstract

Using two independent analyses, it is demonstrated that natural (e.g., estradiol) and some xenoestrogens (e.g., methoxychlor metabolite) are characterized by a lipophilic region which is absent in non-estrogens as well as in phytoestrogens. It is suggested that this lipophilic region affects binding to specific receptors and may in fact serve to differentiate "harmful" from "beneficial" estrogens.

Introduction

Recently, we identified a 6Å 2-dimensional distance (2D) descriptor associated with the carcinogenicity in mice of estrogens (e.g., diethylstilbestrol and 17β-estradiol) (*1*). This descriptor (biophore) was originally recognized in the course of structure-activity relationship (SAR) studies of diethylstilbestrol (*2*) and tamoxifen and toremifene (*3*) using the SAR expert systems CASE and MULTICASE. This biophore was derived from the CASE/MULTICASE learning set of murine carcinogens (*4,5,6,7,8*). Based upon its presence in carcinogenic estrogens we suggested that the 2D biophore represented a ligand binding site on an estrogen receptor (*1*). This hypothesis was supported by the realization that the biophore was derived from estrogens in the carcinogenicity database and the fact that CASE/MULTICASE had been programmed to recognize 2D biophores possessing lipophilic centers as well as moieties capable of hydrogen bonding. These are characteristics which are associated with ligands that bind to cellular receptors. Hence this is consistent with an estrogen possessing a hydrogen-bonding moiety at one end and a lipophilic moiety on the other. In fact, CASE/MULTICASE identified it as a lipophilic anchored para-substituted phenol moiety (e.g., see Figure 1). It should be noted that this 2D descriptor is absent from the vast majority of non-estrogens.

Using CASE/MULTICASE, we identified a number of chemicals, including many estrogens and xenoestrogens, which possess this 2D moiety (see Table 1), as well as a number of estrogens lacking it. However, some of the estrogens devoid of this moiety acquire it following metabolic activation, e.g. tamoxifen metabolism to 4-hydroxytamoxifen, the latter is the metabolite thought to be responsible for the estrogenicity of the parent molecule (*9*). On the other hand, phytoestrogens, as a group, lack this descriptor (Table 1). These findings suggest that the presence of the 2D descriptor could be used to classify estrogens with respect to possible risk to humans and to the ecological biota or even to distinguish between harmful (xenoestrogens) and potentially beneficial estrogens (e.g., phytoestrogens).

While we do not expect this 2D biophore to provide a unifying principle accounting for the action of estrogens, it might provide further insight into their mechanism of action. In the present study we expand further the definition of the 2D biophore, especially with respect to its putative lipophilicity.

Methods

The CASE/MULTICASE methodologies have been described on a number of occasions (*10, 11*). As mentioned earlier the 6Å moiety identified by CASE/MULTICASE involves phenol substitution at the para-position with a carbon atom. The specific lipophilicity of the para-substituent is specified by CASE/MULTICASE to include carbon atoms which are four bonds away from heteroatoms. By this criteria 17β-estradiol was identified as possessing the appropriate lipophilic moiety while the carbon para to the phenol in genistein was found to lack it. To clarify the lipophilicity of the 2D biophore we analyzed a group of molecules with Molecular Modeling Pro (MMP) (*12*) for the presence, location and characteristics of their lipophilic regions.

Briefly, MMP assigns values for the lipophilicity of each atom of a molecule using the procedure ofHansch and Leo (*13*). For example a value of 0.23 is assigned to hydrogens, 0.13 to carbons with one hydrogen, 0.22 to carbons with two or more hydrogens = 0.22, -1.14 to hydroxyl groups and -2.24 to keto oxygens. Each atom is also modified by its neighbors. The value of atoms alpha are multiplied by 0.5, beta by 0.25, gamma by 0.125 and delta by 0.0625. These values are totaled and added to the value of the atom of interest. After all calculations are completed atoms with negative numbers are designated as "hydrophilic" and positive ones as "lipophilic". MMP then colors each atom to denote its degree of lipophilicity or hydrophilicity (see legend Figure 1)

Results and discussion

As mentioned previously, not all estrogens contain the 2D biophore (Table 1). The simplest molecule that contains this biophore is 4-methylphenol (Figure 1) and it can serves to illustrate the biophore. The 1-position of 4-methylphenol contains the hydroxyl group which is both hydrophilic and capable of hydrogen bonding. The 4-position is occupied by a benzylic methyl group which is in a lipophilic environment. In general, the benzylic carbon can be methyl, methylene, methine, or quaternary. Between the para-hydroxyl group and the lipophilic moiety there is a conjugated six-membered ring system which may be substituted at some positions (*1*). The structure of 4-methylphenol can be superimposed on other molecules for easy identification of the 2D biophore.

The major aim of this investigation was to visualize and confirm, using MMP, that, in fact, the MULTICASE biophore is indeed anchored in a lipophilic region. This is readily demonstrated (Figure 1). It should be noted that all the chemicals shown in Figure 1 possess the physical distance requirements of the biophore (i.e., 6Å from phenol to benzylic carbon), however the chemicals lacking the biophore have a benzylic carbon atom located in a region that is either hydrophilic or only somewhat lipophilic. For example diethylstilbestrol and 17β-estradiol, which possess the 2D biophore, have a large lipophilic region that encompasses the para-substituted carbon. On the other hand, dietary estrogens such as coumestrol and genistein, which lack the biophore, have the corresponding carbon embedded in a region intermediate between lipophilic and hydrophilic (Figure 1).

In order for chemicals to have a lipophilic area at the alkyl end of the 2D biophore, heteroatoms (e.g., oxygen atoms) must be sufficiently distant from the para-carbon. Thus, chemicals such as the dietary estrogens with their intra- and extra cyclic oxygens produce an environment which is not very lipophilic and hence the biophore is absent.

As stated earlier, the 2D biophore was originally identified from a carcinogenicity database. The dichotomy between estrogens that display a lipophilic center and those that do not may in fact separate harmful (e.g., carcinogenic) from beneficial (or at least benign) estrogens. Thus carcinogenic estrogens

or their metabolites (e.g., diethylstilbestrol, tamoxifen, and 17β-estradiol) possess the 2D biophore and have independently been shown using MMP to be lipophilic. Additionally xenoestrogens such as the metabolites of methoxychlor, polychlorinated biphenyls and polycyclic aromatic hydrocarbons also possess this biophore and in fact have a lipophilic region to anchor the biophore (Figure 1). On the other hand, dietary phytoestrogens (e.g., genistein, coumestrol, etc.), some of which are thought to be cancer chemopreventive agents, lack this biophore and have been shown herein to lack the lipophilic region (Figure 1).

The 2D biophore and associated lipophilic region appear to have biological significance and are not random occurrences among estrogenic chemicals. Indeed, the lipophilic region associated with the 2D biophore may modulate the binding affinities for these estrogens at different ligand-binding sites (e.g., estrogen receptor or estrogen metabolizing enzymes).

The current report confirms that the 2D biophore describes a lipophilic center. This biophore is able to distinguish between some "beneficial" (e.g., genistein and other phytoestrogens) and some "harmful" (e.g., diethylstilbestrol) estrogens. The ability of this biophore to differentiate estrogens suggests that estrogens elicit their responses through various mechanism. Moreover, this dichotomy suggests that some estrogenic responses may be distinguishable from carcinogenic responses resulting from the action of the estrogens, since not all estrogens are carcinogens. The lipophilic moiety described herein may be involved in this dichotomy.

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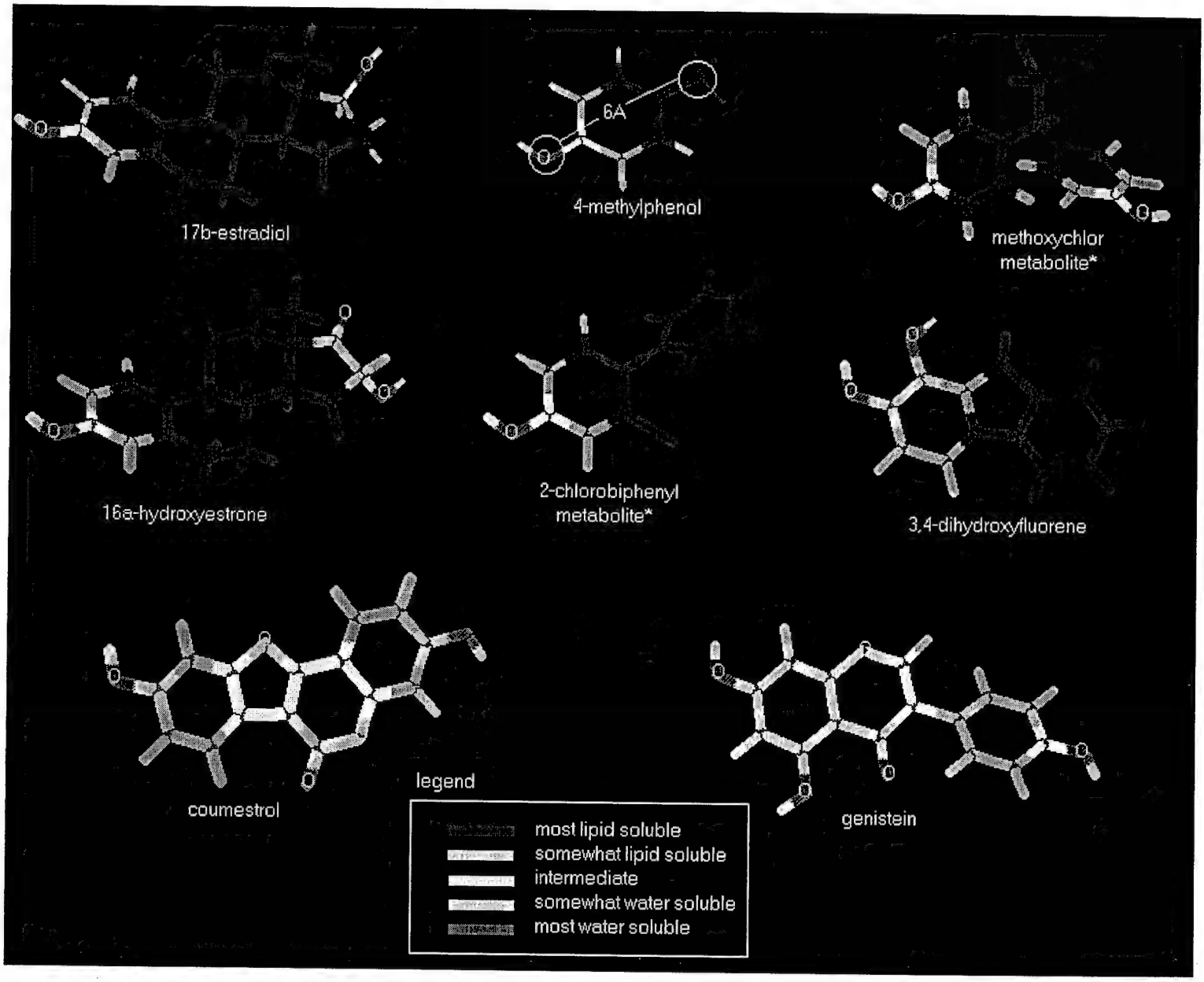

Figure 1. Estrogenic chemicals painted according to lipophilicity. The 6Å 2-dimensional distance biophore is illustrated in 4-methylphenol. All chemicals shown possess the physical distance requirements of the biophore. *Methoxychlor metabolite = 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane; 2-chlorobiphenyl metabolite = 2-chloro-4-hydroxybiphenyl.

Table 1. Distribution of the 2D biophore among selected estrogenic and antiestrogenic chemicals.

chemical	type	2D
Phytoestrogens:		
2',4,4',6'-tetrahydroxydihydrochalcone (phloretin)	phytoestrogen	-
5,7-dihydroxyflavone (chrysin)	phytoestrogen	-
3,5,7- rihydroxyflavone (galangin)	phytoestrogen	-
4',5,7-trihydroxyflavone (apigenin)	phytoestrogen	-
3,3',4',7-tetrahydroxyflavone (fisetin)	phytoestrogen	-
3',4',5,7-tetrahydroxyflavone (luteolin	phytoestrogen	-
3,4',5,7-tetrahydroxyflavone (kaempferol)	phytoestrogen	-
3,5,7-trihydroxy-4'-methoxyflavone (kaempferide)	phytoestrogen	-
3,3',4',5,7-pentahydroxyflavone (quercetin)	phytoestrogen	-
2',3,4',5,7-pentahydroxyflavone (morin)	phytoestrogen	-
4',5,7-trihydroxyflavanone (naringenin)	phytoestrogen	-
3',5,7-trihydroxy-4'-methoxyflavanone (hesperetin)	phytoestrogen	-
3,3',4',5,7-pentahydroxyflavanone (taxifolin)	phytoestrogen	-
4',7-dihydroxyisoflavone (diadzein)	phytoestrogen	-
4',5,7,trihydroxyisoflavone (genistein)	phytoestrogen	-
5,7-dihydroxy-4'-methoxyisoflavone (biochanin A)	phytoestrogen	-
coumestrol	phytoestrogen	-
4,4'-dihydroxystilbene	phytoestrogen	+
α-sitosterol	phytoestrogen	-
zearalenone	phytoestrogen	-
indenestrol A	phytoestrogen	+
tetrahydrocannabinol	phytoestrogen	-
Xenoestrogens and therapeutics		
o,p'-DDE	xenoestrogen	-
chlordecone	xenoestrogen	-
diethylstilbestrol	estrogen	+
4',4"-diethylstilbestrol quinone	estrogen	-
tamoxifen	antiestrogen	-
3-hydroxytamoxifen	antiestrogen	-
4-hydroxytamoxifen acid	antiestrogen	+
toremifene	antiestrogen	-
4-hydroxy-deamino-hydroxytoremifene	antiestrogen	+
ICI 164,384	antiestrogen	+
ICI 182,780	antiestrogen	+
LY 117018	antiestrogen	-
MER 25	antiestrogen	-
17β-estradiol	estrogen	+
17α-ethinyl estradiol	estrogen	+
benzestrol	estrogen	+
dienestrol	estrogen	+
estriol	estrogen	+
estrone	estrogen	+
hexestrol	estrogen	+
megestrol	estrogen	-
norgestrol	estrogen	-
norlestrin	estrogen	+
phenol red	xenoestrogen	-

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ORIGINAL INVESTIGATION

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The carcinogenicity of diethylstilbestrol: structural evidence for a non-genotoxic mechanism

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Abstract An analysis of the structure of diethylstilbestrol (DES) indicates that neither DES nor any of its metabolites are potential mutagens. Moreover, the present analyses suggest (a) that the observed carcinogenic spectrum of DES reflects the activity of metabolic intermediates and (b) that the carcinogenicity of DES in mice is due to the presence of a 6 Å geometric descriptor that appears to be related to an estrogen receptor.

Key words Diethylstilbestrol · Structure · Carcinogenic

Introduction

Diethylstilbestrol (DES) is a recognized rodent as well as human cancer-causing agent (reviewed by IARC 1987a; Marselos and Tomatis 1992), the mechanism of action of which remains the subject of some controversy. Although DES has been reported to induce a number of cytogenetic effects (IARC 1987b), its lack of "structural alerts" for DNA reactivity and its lack of mutagenicity in *Salmonella* as well as its low electronegativity have been taken as evidence that it is a "non-genotoxic" carcinogen (Ashby and Tennant 1991; Ashby and Paton 1993). Additionally, a weight of evidence analysis of the results of short-term tests has also concluded that DES is "non-genotoxic" (Rosenkranz et al. 1986). On the other hand, the recent report that exposure of Syrian hamsters to DES results in the formation of DNA "adducts", detectable by the ^{32}P-postlabeling technique, suggests either that these DNA adducts are "I-compounds" (i.e. endogenous adducts) resulting from DES-induced hormonal stress (Liehr et al. 1986) or that indeed DES or one of its metabolites acts by a "genotoxic" mechanism (see Bhat et al. 1994). It is to be noted, however, that using similar procedures, Montandon and Williams (1994) did not find evidence for DES-induced adducts in either treated rats or Syrian hamsters. An understanding of whether DES causes cancer as a result of a genotoxic or a non-genotoxic mechanism relates to the extent of the risk to humans of exposure to DES as well as to other estrogens, including xenoestrogens.

Thus, if DES is genotoxic, it could, for example, "initiate" cancer in young women, and subsequent environmental, occupational and/or lifestyle factors might act as promoters. On the other hand, as a non-genotoxicant, DES could act as a "promoter" of previously "initiated" women. The second scenario is considered to present less risk to exposed women than the former.

In order to elucidate these different possibilities, we undertook a study of the potential basis of the carcinogenicity of DES and its metabolites based upon structural features recognized as associated with mutagenicity in *Salmonella* and carcinogenicity in rodents.

Materials and methods

Expert system: case/multicase

The CASE methodology has been described on a number of occasions (Klopman 1984; Klopman and Rosenkranz 1984). For the present investigation, we used the recently developed MULTICASE (MC) program (Klopman 1992; Klopman and Rosenkranz 1994). Basically, MC selects its own descriptors automatically from a learning set composed of active and inactive molecules. The descriptors are readily recognizable single, continuous structural fragments that are embedded in the complete molecule. The descriptors consist of either activating (biophore) or inactivating (biophobe) fragments. Each of these fragments is associated with a confidence level and a probability of activity which is derived from the distribution of these biophores and biophobes among active and inactive molecules.

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Upon completion of the fragment analyses, MC selects the most important of these fragments as a biophore, i.e. the functionality that is responsible for the experimentally observed activity of the molecules that contain it. MC then selects the molecules containing this biophore for use as a learning set to identify the chemical properties (i.e. structural fragments) or physical chemical properties (e.g. log P, water solubility, quantum mechanical parameter such as HOMO and LUMO, etc.) that modulate the activity of the initially identified biophore. This process generates a QSAR equation for this subset of molecules. If the data set is congeneric, then the single biophore and associated modulators may explain the activity of the entire training set; however, this will usually not occur and there will be a residue of molecules that are not explained by the single biophore and modulators. When this happens, the program will remove from consideration the molecules already explained by the previous biophore and will search for the next biophore and associated modulators. The process continues until the activity of all the molecules of the learning set have been explained.

The list of biophores identified by MC is then used to predict the activity of yet untested molecules. Thus, upon submission for evaluation, MC will determine whether or not an unknown molecule contains an identifiable biophore. In its absence, the molecule will be predicted to be inactive unless it contains a group that resembles chemically one of the biophores, in which case it will be flagged. When the molecule contains a biophore, the presence of modulators for that biophore will be investigated. MC will then make qualitative as well as quantitative predictions of the activity of the unknown molecule.

Obviously, while biophores are the determining structures, the modulators may determine whether and to what extent the biological potential of the chemical is expressed.

Additionally, MC incorporates selection rules to identify two-dimensional distance descriptors based upon the presence of lipophilic centers. Initially, heteroatoms and lipophilic carbon atoms are designated as "special" atoms. A carbon atom is designated as a lipophilic center if it is at least four bonds away from a heteroatom and it is also the furthest carbon away from the heteroatom when its neighbors are considered. After all the "special" atoms are identified, the distances between all possible pairs are calculated. The distribution of these descriptors among active and inactive molecules is analyzed for statistical significance. Various atom groupings are also investigated, i.e., hydrogen bond acceptors and donors as well as halogens.

Expert system: META

The expert system "META", a computer based metabolism program, was employed to generate putative metabolites of DES. The META program has been recently described in detail (Klopman et al. 1994; Talafous et al. 1994.) META contains a knowledge set of 665 enzyme-catalyzed reaction rules including most of the phase I and II enzyme reactions and 286 spontaneous reactions. When presented with the structure of a parent molecule, META describes a series of metabolic pathways.

For this investigation DES was "metabolized" by META through what can be visualized as four branches, i.e., DES was metabolized through one iteration which yielded four metabolites; two of the metabolites were unconjugated to sulfonic or glucuronic acids and were metabolized for four additional metabolic iterations.

Databases

Salmonella mutagenicity database (SMDB)

The National Toxicology Program (NTP) SMDB was generated under the aegis of the US NTP (Haworth et al. 1983, 1989; Mortelmans et al. 1984, 1986; Lawlor et al. 1985; Zeiger and Haworth 1985; Zeiger et al. 1985, 1987, 1988; Cater et al. 1986; Zeiger 1987, 1990; Ashby and Tennant 1991). The database consists of 1354 chemicals of which 482 are mutagens, 26 are marginal mutagens and 846 are non-mutagens.

Rodent carcinogenicity database

The rodent carcinogenicity database was also generated under the aegis of the US NTP (summarized in Ashby and Tennant 1991). In that database, chemicals of known purity were tested under coded conditions in a standardized two year bioassay. Chemicals were tested at the maximum tolerated dose (MTD) in addition to lower doses. After serial sacrifices or at the termination of the bioassays, complete gross and microscopic analyses of the rodent tissues were performed. The interpretation of the results were reviewed by an external panel of experts.

For the purpose of the present analyses we used the summaries of the bioassays on 301 chemicals (Ashby and Tennant 1991). Due to previously described limitations (Rosenkranz and Klopman 1990), only 287 chemicals were suitable for analysis by MC. Subsequently, due to the ambiguous nature of chemicals classified as equivocal carcinogens, these were also removed from the database. This left a total of 255 chemicals for analysis.

The chemicals that were subjected to analyses were assigned potency values in CASE units. These values are not related to the dose needed to induce cancer, i.e., in contrast to the TD_{50} values (see below). Rather, they reflect the carcinogenic spectrum, i.e., from trans-species carcinogens to carcinogens active in only a single tissue of a single sex of a single species. The classification and CASE unit designations are as follows:

A: Agents carcinogenic to rats and mice at one or more sites – assigned 60 CASE units.
B: Agents carcinogenic only to the rat or mouse at two or more sites – assigned 50 CASE units.
C: Agents carcinogenic only to the rat or mouse at a single site in both sexes – assigned 40 CASE units.
D: Agents carcinogenic at only a single site in a single sex of a single species – assigned 30 CASE units.
E: Agents adequately tested for which equivocal evidence of carcinogenicity was obtained – assigned 20 CASE units; deleted from database.
NC: Agents adequately tested and concluded to be non-carcinogenic – assigned 10 CASE units.

Carcinogenic potency database (CPDB)

The (CPDB) was assembled by Gold et al. (1984, 1986, 1987, 1990, 1993). Two subsets were derived from this compilation (rodent and mouse). In contrast to the NTP rodent carcinogenicity database, this compilation is based primarily on published reports. Presumably, these were not subjected to the rigorous quality assurance and peer review process of the NTP. However, this database has two advantages with respect to the present study:

a. For chemicals judged to be carcinogenic, the dose required for 50% of the animals to remain cancer free is calculated (TD_{50}, which accounts for spontaneous cancer) (Gold et al. 1984; Peto et al. 1984). This provides a more characteristic measure of potency, i.e., the amount of chemical needed to induce cancer in a species.

b. The number of chemicals included in this database is greater than that included in the NTP database. This results in an increased informational content of the database (Rosenkranz et al. 1991; Takihi et al. 1993), resulting in substantial refinements in the nature of the structural determinants.

For each database, all reported dosages were transformed into gavage equivalents. Additionally, the TD_{50} values in mg/kg per day were converted into mmol/kg per day. Using the proper equations (below) chemicals were assigned to activity groups. Chemicals in the range of 10–19 CASE units are inactive or exhibited negligible activity. Chemicals with activities in the range of 20–29 CASE units are marginally active and chemicals in the range of 30–99 CASE units are carcinogenic.

The rodent and mouse carcinogenic potency databases relevant to each MC analysis are discussed below in greater detail.

Rodent carcinogenic potency database The rodent CPDB consists of 437 chemicals, 265 of which are active, 8 are marginally active and 164 are inactive. To be included in this database, a chemical had to have been tested in both rats and mice. To designate potencies in CASE units, the chemicals reported by the authors to be non-carcinogenic in rodents were assigned 10 CASE units. In addition, those chemicals with a TD_{50} value in excess of 28 mmol/kg per day were added to this category. If the chemical was found to be carcinogenic in both rats and mice, the value for the more sensitive species, i.e., the lower TD_{50} value, was used.

For the purpose of the SAR analyses, TD_{50} values (i.e. potencies) in mmol/kg per day were transformed into CASE units using the following relationship:

$$\text{CASE units} = 18.3279 * \log 1/TD_{50} + 46.5517 \qquad \text{(Eqn 1)}$$

Using Equation 1, chemicals in the range of 10–19 CASE units are inactive or exhibit negligible activity. Chemicals with activities in the range of 20–29 CASE units are marginally active and chemicals in the range of 30–99 CASE units are carcinogenic.

Mouse carcinogenic potency database The mouse CPDB consists of 639 chemicals, 291 of which are active, 11 are marginal and 337 are non-carcinogenic. Chemicals reported by the authors to be non-carcinogenic in mice were assigned 10 CASE units along with chemical with TD_{50} value in excess of 51 mmol/kg per day.

For the purpose of the SAR analyses TD_{50} values (i.e. potencies) in mmol/kg per day were transformed into CASE activity units using the following relationship:

$$\text{CASE activity} = 14.1329 * \log 1/TD_{50} + 44.1329 \qquad \text{(Eqn 2)}$$

Using Equation 2, chemicals in the range of 10–19 CASE units are inactive or exhibit negligible activity. Chemicals with activities in the range of 20–29 CASE units are marginally active and chemicals in the range of 30–99 CASE units are carcinogenic.

Results and discussion

Fifteen metabolites of DES were identified from published reports and another 126 putative metabolites were generated by the expert program META. The details of the META analysis and a list of the metabolites are available to readers from Cunningham and Rosenkranz (1994). Based upon the *Salmonella* mutagenicity database, neither DES nor any of its known or putative metabolites were predicted to be a mutagens (Cunningham and Rosenkranz 1994). Thus, DES metabolism presumably does not result in DNA-reactive intermediates, suggesting that DES is non-genotoxic. It should be noted, however, that a similar study of the metabolites of tamoxifen led to the identification of putative metabolites that are mutagenic (Cunningham and Rosenkranz 1995; Cunningham et al. 1995).

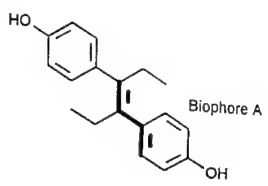


Fig. 1 MultiCASE predictions of the carcinogenicity of DES based upon the National Toxicology Program Rodent Carcinogenicity Database. The biophore (A) associated with rodent carcinogenicity is shown in bold. It is derived from five carcinogenci molecules in the data base (C.I. basic red no. 9; polybrominated biphenyl; tetrachlorvinphos; dichlorodiphenyldichloroethylene; 2-biphenylamine HCl). The molecule contains the biophore (number of occurrences = 4):
Biophore A: CH = C
\
C = C
5 out of the known 5 molecules (100%) containing such a biophore are carcinogens with an average activity of 50. (c.l. = 97%). The following modulator is also present: Constant is 38.7, water solubility = − 1.22, WS Contribution is 2.8. The probability that this molecule is a NTP rodent carcinogen is 85.7%. The compound is predicted to be very active and the projected carcinogenic spectrum is 41 CASE units (38.7 + 2.8).

Examination of the parent molecule, DES, in a variety of carcinogenicity data bases led to the prediction that DES is a rodent carcinogen. Thus, based upon the NTP Rodent Carcinogenicity Data Base, DES is predicted to be carcinogenic (Fig. 1) by virtue of the presence of biophore A which originates from five carcinogenic molecules present in the data base ($p = 0.03$). The predicted "potency" (41 CASE units), indicates (see above) that the chemical has a potential for being carcinogenic to single species at multiple sites in both sexes.

Based upon CPDB, DES is also predicted to be a rodent carcinogen. This prediction is based upon biophore B (Fig. 2) which, in fact, is an elaboration of biophore A derived from the NTP database. Biophore B is present in 13 molecules, 11 of which are carcinogens ($p = 0.01$). The projected activity of 97 CASE units for biophore B is equal to a TD_{50} value of 0.57 mmol/kg per day.

The CPDB analysis also revealed that the majority of DES metabolites are predicted to be carcinogenic by virtue of the presence of biophore B, and moreover, the projected TD_{50} values remain in the same range as that calculated for DES (Fig. 2).

Similarly, based upon the NTP data base, the majority of the DES metabolites are predicted to be carcinogenic. However, a number of metabolites were projected to have increased "potencies" (as expressed in the projected CASE units) which, in the instance of the NTP data base, suggests a broadened carcinogenic spectrum. Thus, the known metabolite 3′3″-dimethoxy-E-diethylstilbestrol (Fig. 3) and the META generated putative metabolite S23 (Fig. 4) are projected to have potencies of 57 CASE units, thereby indicating

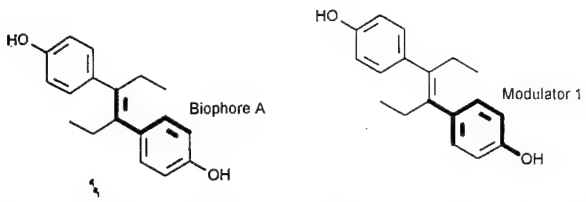
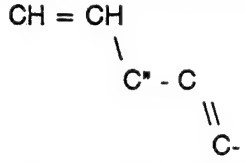
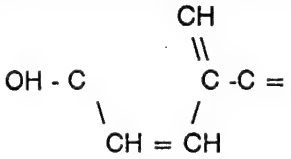


Fig. 2 MultiCASE prediction of carcinogenicity of DES based upon the Carcinogen Potency Data Base of Gold *et al.* (1984, 1986, 1987, 1990, 1993) (CPDB). The probability is derived from the fact that biophore A is present in 13 chemicals in the data base, 11 of which are carcinogens. The presence of biophore A is associated with 61 CASE units of potency. The presence of four copies of modulator 2 contributes a further 39.5 CASE units to the potency. The projected potency (97 CASE units) corresponds to a TD_{50} of 0.57 mmol/kg per day. The molecule contains the biophore (number of occurrences = 4): biophore A:



11 out of the known 13 molecules (85%) containing such biophore are carcinogens with an average activity of 50 (c.l. = 99%). The following modulators are also present: contant is 61.0, modulator 1:



activating 39.5, water solubility = − 1.22 water solubility contribution is − 3.5, The probability that this molecule is a CPDB carcinogen is 80.0%. The compound is predicted to be extremely active and the projected carcinogenicity is 97 CASE units

a potential for inducing cancers in mice and rats at multiple sites (see above). Indeed, this is the characteristic carcinogenic spectrum of DES (IARC 1987a). These results indicate that the trans-species carcinogenic activity associated with DES, may, in fact, be derived from some of its metabolites.

Analysis of DES and its metabolites using the mouse CPDB resulted in the prediction that DES is carcinogenic by virtue of the presence of a geometric descriptor of 6.0 Å (Fig. 5). The 6.0 Å geometric descriptor has also been associated with estrogenicity and anti-estrogenicity (Rosenkranz et al. 1996). This biophore could possibly represent a ligand for an estrogen binding site. Most of the metabolites of DES retain this descriptor (Cunningham and Rosenkranz 1994).

The present study suggests that neither DES nor any of its metabolites are "genotoxicants" as judged by their potential to induce mutagenicity in *Salmonella*. Moreover, it should be noted that one of the putative DES metabolites, i.e. DES-2,3-oxide, is predicted by CASE to be non-mutagenic despite the presence of the epoxide, a putative "structural alert" for DNA reactivity (Ashby and Tennant 1991). In fact, this lack of mutagenicity has been confirmed experimentally (Glatt et al. 1979). The present study reinforces the notion that

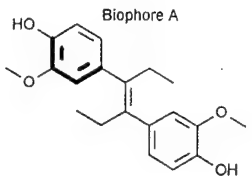
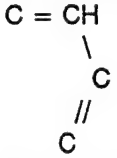


Fig. 3 MultiCASE prediction for 3′.3″-dimethoxy-E-diethylstilbestrol based on the NTP rodent carcinogenicity database. A spectrum of 57 CASE units indicates a potential for inducing cancers in multiple species at multiple sites in both sexes.



The molecule contains the biophore (number of occurrences = 2): biophore A: 35 out of the known 47 molecules (74%) containing such biophore are rodent carcinogens with an average activity of 38 (c.l. = 100%). The following modulator is also present: constant = 56.4, water solubility = − 0.26, WS contribution = 0.6. The probability that this molecule is a rodent carcinogen is 73.5% and the carcinogenic spectrum is projected to be 57 CASE units

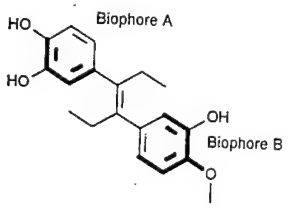
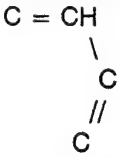
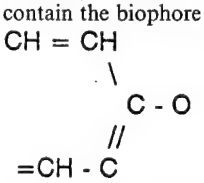


Fig. 4 MultiCASE prediction of the carcinogenicity in rodents of metabolite s23. The molecules contains the biophore (number of occurrences = 2): biophore A:



35 out of the known 47 molecules (74%) containing such a biophore are rodent carcinogens with an average activity of 38 (c.l. = 100%). The following modulator is also present: constant is 56.4 water solubility = 0.44, WS contribution is − 1.0. The molecules also contain the biophore (number of occurrences = 1): biophore B:



7 out of the known 7 molecules (100%) containing such a biophore are NTP CDB carcinogens with an average activity of 51 (c.l. = 99%). The probability that this molecule is a rodent carcinogen is 73.5% due to the first biophore, increased to 86.3% due to presence of the extra biophore. The activity is projected to be 57 CASE units

DES is carcinogenic by virtue of a non-genotoxic mechanism.

Based upon the SAR model described herein, we have demonstrated that many estrogens and anti-estro-

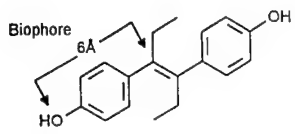


Fig. 5 MultiCASE prediction for diethylstilbestrol in the mouse carcinogenic potency database. The molecule contains the biophore: biophore: 2D fragment [C-] <--6.0 Å--> [OH-] 14 out of the known 16 molecules (87%) containing such biophore are mouse carcinogens with an average activity of 47 (c.l. = 100%). Constant = 51.8. The probability that this molecule is a mouse carcinogen is 83.3% and the activity is projected to be 52 CASE units

gens contain the 6.0 Å geometric descriptors found in DES (Rosenkranz et al. 1996). Thus, it could well be that this biophore identifies the structural basis of the carcinogenicity of DES and of other chemicals which contain it (e.g. ethynylestradiol), thus providing direct structural evidence that the carcinogenicity exhibited by these non-genotoxic agents derives from their estrogenicity. The present study also establishes that the observed carcinogenic spectrum of DES is probably a reflection of the activity of its metabolites as evidenced by the fact that, while the parent molecule is predicted to have a narrow spectrum of carcinogenic activities, its metabolites are predicted to have a much broader activity. This, in fact, reflects the results of animal bioassays (IARC 1987a).

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Identification of a 2-D geometric descriptor associated with non-genotoxic carcinogens and some estrogens and antiestrogens

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A distance descriptor (6Å) originally associated with non-genotoxic mouse carcinogens has been found to be present in some, but not all, estrogens and antiestrogens. It is hypothesized that this descriptor describes a ligand binding site on an estrogen receptor. Evidence is presented that those estrogens and antiestrogens not containing the 6Å distance bind to a different receptor. It is conceivable that binding to the receptor that recognizes the 6Å distance is associated with carcinogenicity.

Introduction

The extension of SAR methods to the elucidation of the action of non-genotoxic carcinogens is controversial. Thus, based upon the electrophilic theory of cancer causation (Miller and Miller, 1977) there is agreement that SAR approaches are useful for studying genotoxic carcinogens. There are, however, differences as to the appropriateness of applying SAR methods to the study of non-genotoxic carcinogens. The perceived obstacle to the successful use of SAR derives from the recognition that there is no single mechanism responsible for the action of non-genotoxic carcinogens (Vainio *et al.*, 1992; Ashby, 1992, 1994; Rosenkranz, 1992). In the present study we identify a geometrical descriptor that appears associated with non-genotoxic carcinogens and which, in fact, may represent a ligand binding site on an estrogen receptor. Moreover, 'genotoxic' carcinogens which are defined operationally as rodent carcinogens that are *Salmonella* mutagens and/or possess 'structural alerts' for DNA reactivity (Ashby and Tennant, 1991) are generally assumed to pose a greater risk to humans than 'non-genotoxic' ones (Ashby and Morrod, 1991). Indeed, the great majority of recognized human carcinogens are 'genotoxic' (Ennever *et al.*, 1987; Shelby, 1988; Bartsch and Malaveille, 1989). The major exception to this generalization are the hormonal carcinogens [e.g. diethylstilbestrol(DES), estradiol] which are thought to act by receptor-mediated mechanisms (Barrett, 1992; Lucier, 1992; IARC, 1992). However, the recent reports that DES (Gladek and Liehr, 1989; Williams *et al.*, 1993) and tamoxifen (Han and Liehr, 1992; White *et al.*, 1992; Hard *et al.*, 1993; Montandon and Williams, 1994) form DNA adducts have generated renewed interest in the basis of the carcinogenicity of this group of agents. Recent studies in our laboratories have been concerned with the structural basis of the carcinogenicity of 'genotoxic' and 'non-genotoxic' carcinogens and their metabolites using the expert systems CASE/MULTICASE and META (Rosenkranz and Klopman, 1995). Indeed, in a recent study of the carcinogenicity of DES and its known and putative metabolites, we were unable to identify either mutagenic or electrophilic metabolites that could be the basis of a 'genotoxic' mechanism of carcinogenicity (Cunningham and Rosenkranz, 1994). In the course of these and related studies, we did, however, obtain evidence that the carcinogenicity of DES in mice was associated with a lipophilic 6Å geometric distance descriptor (Cunningham and Rosenkranz, 1995). Since this could reflect the presence of a ligand binding site, we further investigated the nature of the chemicals which contain this descriptor. The results of that study are reported herein.

Materials and methods

Expert system: CASE/MULTICASE

For the present investigation, we used the MULTICASE (MC) program (Klopman, 1992; Klopman and Rosenkranz, 1994). Basically, MC selects its own descriptors automatically from a learning set composed of active and inactive molecules. The descriptors are readily recognizable single, continuous structural fragments that are embedded in the complete molecule. The descriptors consist of either activating (biophore) or inactivating (biophobe) fragments. Each of these fragments is associated with a confidence level and a probability of activity which is derived from the distribution of these biophores and biophobes among active and inactive molecules.

Upon completion of these analyses, MC selects the most important of these fragments as a biophore, i.e. the functionality that is responsible for the experimentally observed activity of the molecules that contain it. MC then, using the molecules containing this biophore, will use them as a learning set to identify the chemical properties (i.e. structural fragments) or physical chemical properties (e.g. log *P*, water solubility, quantum mechanical parameters such as HOMO and LUMO, etc.) that modulate (either augment or decrease) the activity of the initially identified biophore. This will result in a QSAR equation for this subset of molecules. If the data set is congeneric, then the single biophore and associated modulators may explain the activity of the entire training set. This will usually not occur and there will be a residue of molecules not explained by the single biophore and related modulators. When this happens, the program will remove from consideration the molecules already explained by the previous biophore and will search for the next biophore and associated modulators. The process is continued until the activity of all of the molecules of the learning set has been explained.

The resulting list of biophores is then used to predict the activity of yet untested molecules. Thus, upon submission for evaluation, MC will determine if an unknown molecule contains a biophore. If it does not, the molecule will be predicted to be inactive unless it contains a group that chemically resembles one of the biophores, in which case it will be flagged. When the molecule contains a biophore, the presence of modulators for that biophore will be investigated. MC will then make qualitative as well as quantitative predictions of the activity of the unknown molecule.

Obviously, while biophores are the determining structures, the modulators may determine whether and to what extent the biological potential of the chemical is expressed.

Additionally, MC incorporates the following rules to identify two-dimensional distance descriptors based upon the presence of lipophilic centers. These two-dimensional distances are calculated from the molecular structure. Heteroatoms and lipophilic carbon atoms are designated as 'special' atoms. A carbon atom is designated as a lipophilic center if it is at least four bonds away from a heteroatom and is also the furthest carbon away from the heteroatom when its neighbors are considered. After all the 'special' atoms are identified, the distances between all possible pairs are calculated.

The distribution of these descriptors among active and inactive molecules is analyzed for statistical significance. If the atoms at both ends of the distance descriptor are all the same, including the number of attached hydrogens, the biophore is designated an 'exact' descriptor. Various atom groupings are also investigated, i.e. hydrogen bond acceptors and donors as well as halogens.

The molecule contains the Biophore

biophore A: 2D fragment: [C -] <-- 6.0A --> [OH -] conj generic

14 out of the known 16 molecules (87%) containing such a Biophore are Mouse carcinogens with an average activity of 47 CASE unit (conf.level=100%).

Constant is 51.8

The following Modulator is also present:

modulator 1: OH - CH - Activating 34.2

The probability that this molecule is a Mouse carcinogen is 83.3%

The compound is predicted to be EXTREMELY active
The projected Mouse carcin activity is 86.0 CASE units

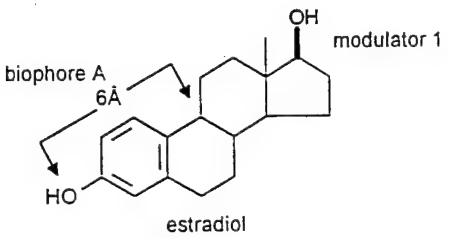

Fig. 1. Prediction of the carcinogenicity in mice of estradiol. The biophore and modulator are indicated. A potency of 86 CASE units indicates a TD_{50} value of 0.001 mmol/kg/day.

The molecule contains the Biophore

biophore A: 2D fragment: [C -] <-- 6.0A --> [OH -] conj generic

14 out of the known 16 molecules (87%) containing such Biophore are Mouse carcinogens with an average activity of 47 CASE units (conf.level=100%).

Constant is 51.8

The following Modulator is also present:

modulator 1: OH - CH - Activating 34.2

The molecule also contains the Biophore:

biophore B: CH2-CH2-CH2-CH -

The probability that this molecule is a Mouse carcinogen is 83.3%
increased to 89.8% due to the presence of the extra Biophore

The compound is predicted to be EXTREMELY active
The projected Mouse carcin activity is 86.0 CASE units

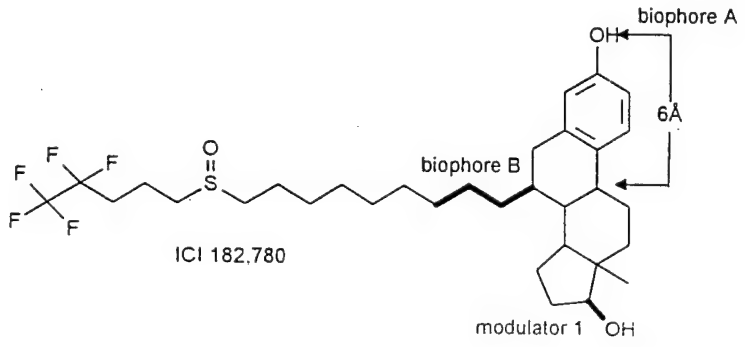

Fig. 2. Prediction of the carcinogenicity in mice of the antiestrogen ICI 182,780. The two structural descriptors are indicated. A potency of 86 CASE units can be translated into a TD_{50} of 0.001 mmol/kg/day.

Table I. Distribution of 6Å descriptors among estrogens, xenoestrogens and antiestrogens

Chemical	Type	6Å Descriptor
o,p'-DDE	Xenoestrogen	–
1-Hydroxy-E-X-diethylstilbestrol	Estrogen metabolite	+
17-α-Ethinyl estradiol	Estrogen	+
2'-Hydroxygenistein	Phytoestrogen	–
3'-Hydroxy-E-diethylstilbestrol	Estrogen metabolite	+
3'-Methoxy-E-diethylstilbestrol	Estrogen metabolite	+
3-Hydroxytamoxifen	Antiestrogen metabolite	–
4',4"-Diethylstilbestrol quinone	Estrogen metabolite	–
4-Hydroxytamoxifen acid	Antiestrogen metabolite	+
4-Hydroxy-deamino-hydroxytoremifene	Antiestrogen metabolite	+
4-Hydroxytoremifene	Antiestrogen metabolite	+
Benzestrol	Estrogen	+
Chlordecone	Xenoestrogen	–
Coumestrol	Phytoestrogen	–
Dienestrol	Estrogen	+
Diethylstilbestrol	Estrogen	+
Estriol	Estrogen	+
Estrone	Estrogen	+
Genistein	Phytoestrogen	–
Hexestrol	Estrogen	+
Indenestrol A	Phytoestrogen	+
Megestrol	Estrogen	–
Mestranol	Estrogen	–
Norgestrel	Estrogen	–
Norlestrin (isomer A)	Estrogen	+
Phenol red	Estrogen	–
Tamoxifen	Antiestrogen	–
Tamoxifen-bis-phenol	Antiestrogen metabolite	+
Tetrahydrocannabinol	Xenoestrogen	–
Toremifene	Antiestrogen	–
Zearalenone	Xenoestrogen	–
β-Estradiol	Estrogen	+
z-Bisdehydrodoisynolic acid	Phytoestrogen	+
Allenolic acid	Phytoestrogen	+
Kaempferol	Phytoestrogen	–
Quercetin	Phytoestrogen	–
ICI 164,384	Antiestrogen	+
ICI 182,780	Antiestrogen	+
LY 117018	Antiestrogen	–
MER 25 (ethamoxytriphetol)	Antiestrogen	–
3-Phenylacetylamino-2,6-piperidinedione	Antiestrogen	–
p-Hydroxy-3-phenylacetylamino-2,6-piperidinedione	Antiestrogen	–

Additional hydroxylated putative metabolites of tamoxifen and toremifene also contain the 6Å structural descriptor.

Expert system: META

The expert system 'META', a computer based metabolism program, was used to investigate the metabolism of tamoxifen and tomerifene. The META program has recently been described in detail (Klopman *et al.*, 1994; Talafous *et al.*, 1994). Briefly, META contains a knowledge set of 665 enzyme-catalyzed and 286 spontaneous reactions which constitute most of the phase I and II metabolic pathways. When presented with the structure of a parent molecule, META indicates a series of possible metabolic pathways which include a graphical description of each possible metabolite(s) and intermediate metabolite(s) as well as the enzymes used to generate these.

Mouse carcinogenic potency database

The carcinogenic potency database was assembled by Gold *et al.* (1984, 1986, 1987, 1990, 1993). A subset of mouse carcinogens (males and females) was derived from this compilation and subjected to MC analysis. In that database, chemicals reported as carcinogenic by the primary authors are accompanied by their TD_{50} values, i.e. the dose required for 50% of the animals to remain cancer-free (Gold *et al.*, 1984; Peto *et al.*, 1984). These were transformed into gavage equivalents (Gold *et al.*, 1984, 1986; Brown and Ashby 1990). Additionally, the TD_{50} values (in mg/kg/day) were converted in mmol/kg/day. Using equation (1) (see below), chemicals were assigned to activity groups.

The database consists of 639 chemicals, 291 of which are carcinogens, 11 are marginal and 337 are non-carcinogens. Chemicals reported by the authors to be non-carcinogenic in mice were assigned 10 CASE units together with chemicals with a TD_{50} value in excess of 51 mmol/kg/day.

For the purpose of the SAR analysis, TD_{50} values (i.e. potencies) in mmol/kg/day were transformed into CASE units using the following relationship:

$$\text{CASE activity} = 14.1329 \times (\log 1/(TD_{50}) + 44.1329. \quad (1)$$

In order to accomodate the broad range of TD values present in the database, we chose to relate the TD_{50} values to CASE units in a logarithmic function. Thus, using equation **1**, chemicals in the range of 10–19 CASE units are inactive or exhibit negligible activity. Chemicals with activities in the range of 20– 29 CASE units are seen as marginally active and chemicals in the range of 30–99 CASE units are called carcinogenic.

It should be noted that since the database is for carcinogenicity in mice (males and females), the MULTICASE predictions do not specify the gender involved. Moreover, in the analyses no attempt was made to predict tissue-specificity.

Table II. Some chemicals containing the 6Å descriptor

11-Hydroxybenzo[*a*]pyrene
1,3,4-Xylenol
2-Amino-4-methylphenol
2,3-Dihydroxynaphthalene
2,4-Dimethylphenol
2,4,6-Trimethylphenol
3-*t*-Butyl-5-methylcatechol
3-*t*-Octyl-5-methylcatechol
3,4,5-Trimethylphenol
3,9-Dihydroxybenzanthrone
4-Hydroxy-desmethyl-toremifene
4-Methyl-2-nitrophenol
4-Methylcatechol
4-Methylphenol (*p*-cresol)
4,4'Dihydroxy-3-(hydroxymethyl) diphenylmethane
4,5-Dimethyl-3-pentadecylcatechol
4,5-Dimethylcatechol
5-Hydroxyacenaphthene
7-Hydroxy-4-isopropyltropolone
Allylsyringol
Aucuparin
Butylated hydroxytoluene (BHT)
CI Disperse Yellow 3
CI Pigment Red 3
Calmagite
CI Pigment Red no. 23
Citrus Red 2
D and C Red no. 10
D and C Red 9
Desoxyhemigossypol
Diflunisal
Eugenol
Fast Green FCF
Gamma-thajaplicin
Gossypol
Hemigossypol
Levallorphan
NSC 377163 (pyrazoloacridine)
Oxymetazoline
p-Phenylphenol
Purpurogallin
Scarlet Red
Violaceol-I
Viridicatumtoxin

The molecules listed above were selected from among a group of 5400 molecules representative of the 'universe of chemicals' because they contain the 6Å descriptor. In this analysis no consideration was given to the possible metabolism of these or the other molecules in the testing set. The 5400 chemicals tested represent a random selection of chemicals taken to reflect the 'universe of chemicals' (National Academy of Sciences, 1984).

Results and discussion

The 6Å descriptor (see Figure 1) was originally identified as a biophore associated with carcinogenicity in mice (Cunningham and Rosenkranz, 1995). That finding was based upon the

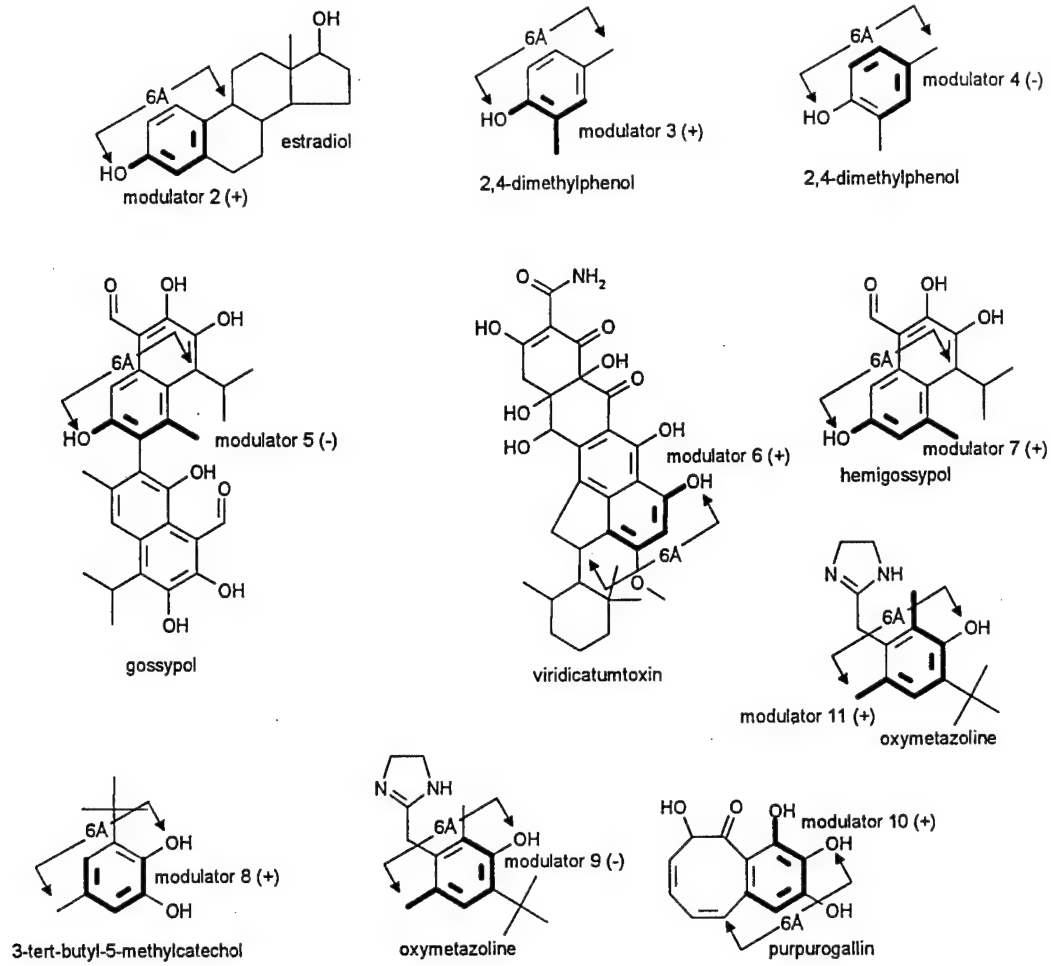

Fig. 3. Description of the modulators associated with the 6Å biophore; (+) or (–) next to the modulator designation indicates an activating or an inactivating modulator, respectively.

Carcinogen Potency Database of Gold *et al.* (1984, 1986, 1987, 1990, 1993). Among the chemicals in the database which contain this descriptor was estradiol and related chemicals (Figure 1), thus suggesting that the 6Å descriptor may be associated with estrogenicity. In order to determine whether this was the case, we tested a series of chemicals reported to be endowed with estrogenicity for the presence of the 6Å descriptor. A number, but not all, of them displayed this property, suggesting that, indeed, this descriptor is related to estrogenicity. It should be noted that while the therapeutically useful anticancer agents tamoxifen and toremifene lack the 6Å descriptor (Table I), some of their metabolites contain it. Indeed, among these are the metabolites which are believed to be responsible for the estrogenicity of tamoxifen (see Lerner and Jordan, 1990). If it is assumed that the 6Å descriptor identifies not only a biophore associated with murine carcinogenicity, but also an estrogen-receptor ligand, then the presence of this moiety in tamoxifen metabolites may explain the carcinogenicity as well as the estrogenicity of the parent molecule. Moreover, this suggests that the two phenomena may result from the same mechanism, i.e. binding to a specific site. If this is so, then it could be advantageous to design antiestrogens which lack the 6Å descriptor and therefore bind to another receptor. In order to determine whether this is a viable alternative, we tested a number of estrogens and antiestrogens. It is of interest to note that some of these (e.g. ICI 164,384 and ICI 182,780) contain the 6Å descriptor (Figure 2) while others (e.g. LY 117018) do not (see Table I). It has already been suggested that LY 117018 and tamoxifen (Black and Goode, 1981; Scholl et al., 1983) and LY 117018 and ICI 164,384/ICI 182,780 (Coradini *et al.*, 1994) have different bases for their antiestrogenicity. The present findings together with the previously reported dichotomy of the action of antiestrogens, suggests that the latter may act by different mechanisms. Our findings further suggest the possibility that the 6Å distance descriptor identifies a ligand binding site on one of the estrogen receptors. If, indeed, the 6Å biophore is associated with rodent carcinogenicity, then perhaps targeting the estrogen receptor which does not recognize this moiety may be a promising endeavor for the development of therapeutically useful antiestrogens.

As mentioned earlier, the 6Å biophore was first identified using a rodent carcinogenicity database which is not congeneric with respect to chemical species. Accordingly, the modulators identified as associated with this biophore may not provide a complete understanding of the factors affecting activity.

In order to further explore the nature of the 6Å biophore, the database was supplemented with a series of diverse molecules containing these descriptors ($n = 31$) as well as with congeners lacking it ($n = 9$). Upon analysis of this 'synthetic' learning set with MULTICASE, the 6Å biophore was once again identified ($P < 0.01$). However, the nature of

the modulators associated with this biophore permitted a further delineation of their nature. Thus, all of the modulators are within the 6Å domain and describe moieties that either enhance or decrease the projected activity (see Figure 3). Moreover, the physical chemical modulator affecting this biophore is the log P (octanol:water partition coefficient) which further substantiates that the 6Å biophore encompasses a lipophilic center. Thus, the modulators are consistent with the possibility that the 6Å biophore describes a ligand which is recognized by a specific receptor.

Finally, in order to gain an understanding of the possible distribution of the 6Å biophore among molecules, we determined its presence among molecules ($n = 5400$) representing the 'universe of chemicals'. Some of the chemicals containing this moiety are listed in Table II and it is of interest that it is present in a wide selection of agents, some of which are in widespread use (e.g. BHT), others which are used in cancer chemotherapy (e.g. NSC377163) and still others which are used in traditional medicine (e.g. hemigossypol). Some of these might be endowed with estrogenicity. It is of further interest that some hydroxylated metabolites of polycyclic aromatic hydrocarbons (e.g. 11-hydroxypyrene, 5-hydroxydibenz(a,h)anthracene, 3-hydroxy-7,12-dimethylbenz[a]anthracene) contain the 6Å biophore (Table II). The estrogenicity of polycyclic aromatic hydrocarbons is a recognized phenomenon (Davis *et al.*, 1993; Safe, 1995).

The current findings suggest a number of additional studies that should lead to a further refinement of the biophore as well as a better understanding of its relevance to the induction of cancer and estrogenicity. Moreover, the recognition of at least two classes of estrogens differing in a geometric descriptor may enable us to identify the nature of the ligand which binds to the other estrogen receptors.

The present report documents the existence of a structural descriptor which appears to be related to non-genotoxic carcinogens. Moreover, the new biophore implies a mechanism of action as well. This indicates the feasibility of applying SAR approaches to non-genotoxic carcinogens.

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MUTATION RESEARCH

Fundamental and Molecular Mechanisms of Mutagenesis

Mutation Research 349 (1996) 85–94

A study of the structural basis of the carcinogenicity of tamoxifen, toremifene and their metabolites

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Abstract

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Abstract

An analysis of the chemical structure of tamoxifen, toremifene and their metabolites indicates that metabolism to a DNA-reactive hydroxylamine intermediate is possible. The parent compounds and many of their metabolites are predicted to be rodent carcinogens. Moreover, many of these metabolites contain a 6 Å or 8.4 Å distance descriptor biophore. These geometric descriptors may be related to an ability of these chemicals to bind to an estrogen receptor. The prediction of the carcinogenicity of toremifene is not in accord with studies published thus far. However, the reports available have not excluded this possibility, since the protocols used have not addressed it systematically.

Keywords: Carcinogenicity; Tamoxifen; Toremifene; Structure-activity; Metabolism

1. Introduction

Estrogens and their antagonists, the antiestrogens, are the subjects of debate regarding aspects of cancer etiology and therapy as well as other endocrine-related health effects. The distinction between estrogen and antiestrogen is obscure. Several of the factors that determine the estrogenicity vs. antiestrogenicity of a chemical are species- and tissue-specific (for a review on antiestrogens, see Lerner and Jordan, 1990).

Antiestrogens, such as tamoxifen (i.e., antiestrogenic in human breast tissue), are pivotal to chemotherapeutic strategies for breast cancer. Newer antiestrogens are being introduced into breast cancer chemotherapeutic regimens such as the tamoxifen congener toremifene and the 'pure' antiestrogens ICI 164,384 and ICI 182,780. Due to a decreased risk of developing contralateral breast cancer in patients receiving tamoxifen (Early Breast Cancer Trialists' Collaborative Group, 1992a, b; Fisher et al., 1989), tamoxifen is presently undergoing trials as a prophylactic agent against breast cancer by the National Surgical Adjuvant Breast and Bowel Project (NSABP).

While antiestrogens are useful in the treatment and possible prevention of breast cancer, recently environmental estrogens have been implicated in the etiology of breast cancer (Davis et al., 1993). Phenols and halogenated hydrocarbons represent two

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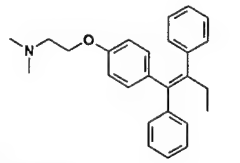
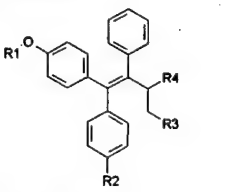
groups of organic chemicals widely used as solvents and industrial intermediates. Human exposures to these chemicals can occur occupationally or environmentally. The toxicological properties of these chemicals include estrogenic and antiestrogenic activities and, accordingly, they may be classified as 'xenoestrogens'. Xenoestrogen exposure is implicated in certain reproductive anomalies in both humans and wildlife (Guillette et al., 1994; Santti et al., 1994; Sharpe and Skakkebaek, 1993). Moreover, if these agents act through a receptor that also recognizes physiological and therapeutic estrogens or their antagonists, it can be expected that they will exhibit at least an additive effect with respect to the activities of the former.

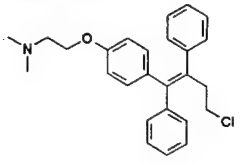
The purpose of the present investigation was to gain insight, through the structure-activity relationships (SAR), of the carcinogenic and therapeutic mechanisms of estrogens and antiestrogens with emphasis on the antiestrogens tamoxifen (TMX) and toremifene (TRM) (Fig. 1). The present study is part of our continuing investigations of the bases of action of 'non-genotoxic' carcinogens (Rosenkranz and Klopman, 1990a; Lee et al., 1995).

TMX and TRM are relevant to human carcinogenesis due to their similarities with diethylstilbestrol (reviewed by IARC, 1987; Marselos and Tomatis, 1992). TMX has been implicated in the induction of rare endometrial cancer in humans (Cohen et al., 1992; Fornander et al., 1989; Gal et al., 1991; Gusburg, 1990). In experimental animals TMX induces hepatocellular carcinomas and hepatic adenomas (Hard et al., 1993a, b; Hirsimaki et al., 1993; Greaves et al., 1993; Williams et al., 1993). It has been suggested that TMX acts as a 'genotoxic' carcinogen due to its ability to form DNA adducts as detected by the ^{32}P-postlabeling technique (Hard et al., 1993a; Han and Liehr, 1992, Montandon and Williams, 1994; White et al., 1992; Phillips et al., 1994) and the induction of micronuclei in cultured cells (Styles et al., 1994; White et al., 1992). These findings have direct relevance to human risk, as 'genotoxic' carcinogens are thought to present a greater risk than 'non-genotoxic' ones (Ashby and Morrod, 1991). In fact, the vast majority of recognized human carcinogens are genotoxicants (Ennever et al., 1987; Bartsch and Malaveille, 1989; Shelby, 1988).

The mechanism of TMX reactivity with DNA has not been elucidated. Styles et al. (1994) have reported that TMX may be metabolized to DNA-reac-







Name	R1	R2	R3	R4
tamoxifen (TMX)	$CH_2CH_2N(CH_3)_2$	H	H	H
N-desMe-TMX[1, 2, 3]	$CH_2CH_2NH(CH_3)$	H	H	H
N-didesMe-TMX[1, 2]	$CH_2CH_2NH_2$	H	H	H
deamino-OH-TMX[1, 2]	CH_2CH_2OH	H	H	H
4-OH-TMX[1, 2 3]	$CH_2CH_2N(CH_3)_2$	OH	H	H
4-OH-N-desMe-TMX[1]	$CH_2CH_2NH(CH_3)$	OH	H	H
4-OH-deamino-OH-TMX[1]	CH_2CH_2OH	OH	H	H
TMX-N-oxide[1]	$CH_2CH_2NO(CH_3)_2$	H	H	H
α-OH-TMX-N-oxide[1]	$CH_2CH_2NO(CH_3)_2$	H	H	OH
α-OH-TMX[5]	$CH_2CH_2NH(CH_3)$	H	H	OH
TMX-bis-phenol[4]	H	OH	H	H
TMX acid[4]	CH_2COOH	H	H	H
TMX-glycine-OH[4]	CH_2CONCH_2COOH	H	H	H
4-OH-TMX acid[4]	CH_2COOH	OH	H	H
Me-4-OH-TMX-acid[4]	CH_2COOMe	OH	H	H
toremifene (TRM)[1]	$CH_2CH_2N(CH_3)_2$	H	Cl	H
N-desMe-TRM[1]	$CH_2CH_2NH(CH_3)$	H	Cl	H
N-didesMe-TRM[1]	$CH_2CH_2NH_2$	H	Cl	H
deamino-OH-TRM[1]	CH_2CH_2OH	H	Cl	H
4-OH-TRM[1]	$CH_2CH_2N(CH_3)_2$	OH	Cl	H
4-OH-N-desMe-TRM[1]	$CH_2CH_2NH(CH_3)$	OH	Cl	H
4-OH-deamino-OH-TRM[1]	CH_2CH_2OH	OH	Cl	H

Fig. 1. Chemical structures of tamoxifen and some metabolites. [1] Berthou and Dreano, 1993; [2] Robinson and Jordan, 1988; [3] Robinson et al., 1991; [4] Ruenitz and Nanavati, 1990; [5] Potter et al., 1994.

tive epoxides. However, α-hydroxylation of the ethyl group yielding an alkylating metabolite has been postulated (Potter et al., 1994) and experimental evidence has been presented in support of this mechanism (Phillips et al., 1994; Randerath et al., 1994a, b).

TRM is also endowed with estrogenic and antiestrogenic properties (Homesley et al., 1993; Stenbygaard et al., 1993, Valavaara et al., 1988; Vogel et al., 1993). Unlike TMX, TRM is reported to be 'non-genotoxic' (i.e., no adducts by ^{32}P-postlabeling technique) (Montandon and Williams, 1994) and to be non-carcinogenic to rodents under conditions in which TMX induces liver tumors in rats (Tucker et al., 1984). Thus comparison of TMX and TRM could permit a determination of the mechanism of carcinogenicity of this group of chemicals, especially with respect to the possibility that their estrogenicity and carcinogenicity are separate phenomena.

2. Methods

2.1. CASE / MULTICASE methodology

The CASE (Klopman, 1984; Klopman and Rosenkranz, 1984) and MULTICASE (MC) (Klopman, 1992; Klopman and Rosenkranz, 1994) methodologies has been described on a number of occasions. Basically, MC selects its own descriptors automatically from a learning set composed of active and inactive molecules. The descriptors are readily recognizable single, continuous structural fragments that are embedded in the complete molecule. The descriptors consist of either activating (biophore) or inactivating (biophobe) fragments. Each of these fragments is associated with a confidence level and a probability of activity that is derived from the distribution of these biophores and biophobes among active and inactive molecules.

Additionally, MC incorporates the following rules to identify two-dimensional distance descriptors based upon the presence of lipophilic centers. These two-dimensional distances are calculated from the molecular structure. Heteroatoms and lipophilic carbon atoms are designated as 'special' atoms. A carbon atom is designated as a lipophilic center if it is at least four bonds away from a heteroatom and is also the farthest carbon away from the heteroatom when its neighbors are considered. After all the 'special' atoms are selected the distances between all possible pairs are calculated. The distribution of these descriptors among active and inactive molecules is analyzed for statistical significance. Various atom groupings are also investigated, i.e., hydrogen bond acceptors and donors as well as halogens.

2.2. Metabolites

A group of experimentally identified metabolites of TMX and TRM was assembled from the literature (Fig. 1). In conjunction with this, the expert system 'META', a computer based metabolism program, was employed (Klopman et al., 1994; Talafous et al., 1994). META contains a knowledge set of 655 enzyme-catalyzed and 286 spontaneous reactions that include most of the phase I and II enzymes. When META is presented with the structure of a parent molecule it indicates a series of possible metabolic pathways and a structural representation of the metabolites.

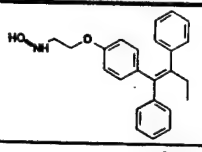
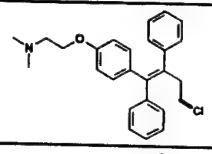
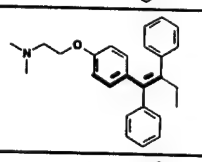
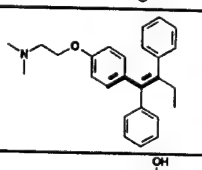
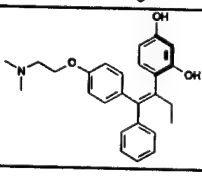
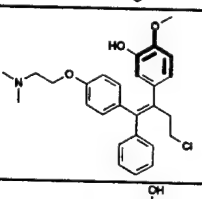
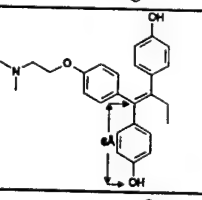
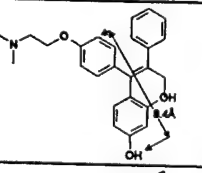
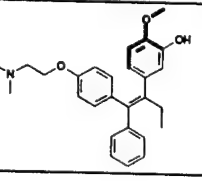
The parent compounds were 'metabolized' through one iteration which yielded a group of metabolites. Each non-conjugated metabolite, was again 'metabolized' and so on through four iterations. Overall, 132 TMX and 182 TRM non-conjugated experimental and putative metabolites were assembled. A description of these putative metabolites can be found elsewhere (Cunningham and Rosenkranz, 1995).

2.3. Databases

TMX, TRM and the set of metabolites were analyzed by MC using carcinogenicity and mutagenicity databases. The rules for inclusion of chemicals in the databases (i.e., learning sets) have been described previously (Rosenkranz and Klopman, 1990a, c).

The *Salmonella* mutagenicity database (SMDB) was generated under the aegis of the U.S. NTP (Ashby and Tennant, 1991; Cater et al., 1986; Haworth et al., 1983, 1989; Lawlor et al., 1985; Mortelmans et al., 1984, 1986; Reid et al., 1984; Zeiger, 1990; Zeiger and Haworth, 1985; Zeiger et al., 1985, 1987, 1988). The database consisted of 1354 chemicals. A SAR analysis of a subset of these chemicals

Table 1
Summary of biophores used in the predictions associated with the carcinogenicity of tamoxifen and toremifene

Database: Biophore	Representative TMX, TRM or metabolite with biophore illustrated	Number of chemicals in database with biophore (active/total)	Probability of activity associated with possession of biophore	Predicted potency range for parent and metabolites
SMDB: HO-NH		4/4	0.83	TMX: 39 TRM: 39
SMDB: $-CH_2-Cl$ CPDB: $-CH_2-Cl$		37/48 20/24	0.76 0.91	TMX: not applicable TRM: 35 TMX: not applicable TRM: 50-70
CPDB: CH=CH-C=C-C=		11/13		TMX: 44-116 TRM: 41-114
NTP CDB CH=C-C=C-		5/5	0.86	TMX: 41-52 TRM: 43-56
NTP CDB: C=CH-C=C		35/47	0.74	TMX: 41-54 TRM: 42-54
NTP CDB: O | CH-CH-C=C-CH=		7/7	0.86	TMX: 57 TRM: 57
MCPDB [C-]<— 6.0 Å —>[OH-]		14/16	0.83	TMX: 52 TRM: 52
RCPDB: [C-]<— 8.4 Å —>[OH-]		7/7	0.89	TMX: 85 TRM: 85
RCPDB: O-CH3 | =C-C=CH-CH=		5/5	0.86	TMX: +85 TRM: not applicable

has been reported previously (Rosenkranz and Klopman, 1990b).

The rodent carcinogenicity database (CDB) was generated under the aegis of the U.S. NTP. For the purpose of the present analyses we used the summaries of the bioassays on 301 chemicals (Ashby and Tennant, 1991). Due to the ambiguous nature of chemicals classified as 'equivocal carcinogens' and chemicals removed because of structural limitations, 255 chemicals were used for the analysis. The six levels of carcinogenicity (A–F) (Ashby and Tennant, 1991) have been converted to 10–60 CASE units, respectively. A SAR analysis of a subset of these chemicals has been reported previously (Rosenkranz and Klopman, 1990c).

The carcinogenic potency database (CPDB) was assembled by Gold et al. (1984, 1986, 1987, 1990, 1993). Three subsets were derived from this compilation (rodent, mouse and rat). In contrast to the CDB, this compilation is based primarily on published reports. Presumably these were not subjected to the same rigorous quality assurance and peer review tiers of the NTP. However this database has two advantages with respect to the present study:

(a) For chemicals judged to be carcinogenic, TD_{50}, the dose required for 50% of the animals to remain cancer free, was calculated (Gold et al., 1984; Peto et al., 1984).

(b) The number of chemicals included in this database is greater than that included in the CDB. This results in an increased informational content of the database as well as allowing refinements in the nature of the structural determinants.

For each database all dosages reported were transformed into gavage equivalents. Additionally, the TD_{50} value in mg/kg per day was converted into mmol/kg per day. Using the appropriate equations (see below) potencies were transformed into CASE units and the chemicals were assigned to activity groups.

The mouse carcinogen subset of the CPDB consisted of 639 chemicals, 291 of which are active, 11 are marginal and 337 are non-carcinogenic. The rat carcinogen subset of the CPDB consisted of 744 chemicals, 380 of which are active, 15 are marginal and 349 are non-carcinogenic.

The rodent carcinogen subset of the rodent CPDB consisted of 437 chemicals, 265 of which are active, 8 are marginally active and 164 are inactive. To be included in this database chemicals had to be tested both in the rat and the mouse. If the chemical was determined to be carcinogenic in both rats and mice the value for the more sensitive species, i.e., the lower TD_{50} value, was used.

For the purpose of the SAR analyses TD_{50} values in mmol/kg per day were transformed into CASE units using the following relationships:

$$\text{CASE unit} = 14.1329 * (\log 1/TD_{50}) + 44.1329 \text{ (mouse CPDB)}$$

$$\text{CASE unit} = 20.124 * (\log 1/TD_{50}) + 44.066 \text{ (rat CPDB)}$$

$$\text{CASE unit} = 18.3279 * (\log 1/TD_{50}) + 46.5517 \text{ (rodent CPDB)}$$

Chemicals in the range of 10 to 19 CASE units are inactive or exhibited negligible carcinogenicity. Chemicals with activities in the range of 20 to 29 CASE units are marginally active and chemicals in the range of 30 to 99 CASE units are carcinogenic.

3. Results

MC analysis using the SMDB indicates that TMX and TRM have a potential for mutagenicity. MC identified the moiety $-CH_2-Cl$ present in TRM and many of its metabolites as an indication that TRM may be mutagenic (Table 1). Examination of the configuration of the $-CH_2-Cl$ fragment among the 37 mutagenic chemicals containing it indicates that their configurations are greatly different from that of TRM. The mutagens that possess $-CH_2-Cl$ are primarily haloalkanes, esterfied phosphates or nitrogen mustards, and act through a DNA alkylating mechanism. Additionally, the $-CH_2-Cl$ moiety may not be always related to the mutagenicity of chemicals that contain it. The mutagenicity of these chemicals may be derived from other functionalities present in the molecule, e.g., nitro functionalities, as in nitrobenzyl chloride and epoxy functionalities, as in 1,2-epoxy-3-chloropropane. In fact, the chemical in the database that most resembles the configuration of the $-CH_2-Cl$ moiety of TRM is the *non-mutagenic* tetrachlorodiphenylethane. (For a list of the chemicals containing the $-CH_2-Cl$ moiety, see Cunningham and Rosenkranz (1995)).

MC identified a hydroxylamine moiety, OH–NH– (Table 1). This biophore is present in several of the META derived metabolites from both TMX and TRM. Considering the presence of this fragment, MC predicted that these metabolites may be mutagenic. Aliphatic hydroxylamines are known mutagens capable of reaction with deoxycytidine (Freese, 1963) while arylhydroxylamines form adducts with the O-6 (Kadlubar et al., 1978) or C-8 (Beland et al., 1983) of deoxyguanosine. Several metabolic pathways were identified by META which produce hydroxylamine metabolites. Details of the metabolic transformations can be found in Cunningham and Rosenkranz (1995). The putative metabolites that contain this biophore are related to desmethyl TMX and desmethyl TRM (Fig. 1). They can be considered aliphatic hydroxylamine analogs. It is noteworthy that a recent report of TMX-induced DNA adducts has identified deoxyguanosine as the primary target (Martin et al., 1995).

Our analyses indicate a pathway for the generation of potential DNA reactive metabolites for TMX and TRM. Whether these explain the adducts detected by the ^{32}P-postlabeling technique is problematic, only TMX and not TRM are reported to give rise to such adducts. Moreover, in the case of DES treatment that yield adducts, it has been shown that they reflect endogenous chemicals which may result from hormonal stress (Liehr et al., 1986).

MC analysis of TMX, TRM and their metabolites identified a series of structural features indicative of potential carcinogenicity in both the CDB and the rodent CPDB (Table 1). The parent compounds, as well as many of the metabolites, posses the biophore CH=C–C=C– identified by the CDB and the closely related biophore, CH=CH–C=C–C= identified by the rodent CPDB (see Table 1). Analyses based on the CDB identified in some metabolites the biophores C=CH–C=C and CH–CH–C(–O)=C–CH=. Lastly, the rodent CPDB-based analyses indicated the $-CH_2-Cl$ moiety of TRM is predictive of carcinogenicity. However, the $-CH_2-Cl$ moiety can be dismissed as related to carcinogenicity for the same reasons it was eliminated as a descriptor for mutagenicity in *Salmonella*.

The projected potency of TMX and TRM based on the rodent CPDB is 48 and 46 CASE units, respectively. These values translate into TD_{50} values of 0.8 and 1.1 mmol/kg per day, which is in the same range as aflatoxin B1 and *N,N*-dimethylaniline. The projected potency based upon the CDB of TMX and TRM is 49 and 51 CASE units, respectively. These values suggest that the parent molecules, TMX and TRM, are carcinogenic to a single species but at multiple sites. Additionally, based upon rodent CPDB, the range of predicted potencies of the metabolites is greater than for the parent molecule (Table 1). The range of potencies in the CDB, respectively, for TMX and TRM metabolites was 41–57 and 42–57 CASE units. These values are derived from two separate biophores. This suggests (see Cunningham and Rosenkranz, 1995) that some of the metabolites may have the potential to be carcinogenic in male and female rats and mice at multiple sites. Moreover, both the increased potencies and extended carcinogenic range suggests that the predicted carcinogenicity of TMX and TRM is due to their metabolites. It should be noted that these predictions are not completely in accord with published reports that indicate that TMX but not TRM is a rat carcinogen. However, TMX is a suspected human carcinogen based on its ability to cause cancer in several animal species. We could not find published reports of TMX and TRM cancer bioassays using an NTP-type protocol in which carcinogenicity to multiple species of both genders and examination of all tissues was included. Hence our predictions of the carcinogenicity of TRM remain to be verified

MC analysis of TMX and TRM in the mouse and rat CPDB found no evidence to predict that the parent molecules are carcinogenic. However, identified as well as putative metabolites of TMX and TRM possess biophores indicative of a potential for carcinogenicity (Table 1). Two of these are distance descriptors of 6.0 Å and 8.4 Å between a lipophilic carbon and a hydroxyl moiety associated with the mouse CPDB and the rat CPDB, respectively. The structural fragment =C $(-OCH_3)$–CH=C=C present in one TMX metabolite is a carcinogenic biophore based upon the rat CPDB.

The putative metabolites of TMX and TRM predicted to be carcinogenic are so by virtue of two-dimensional distance descriptors. The possible significance of these distance descriptors may be related to the physical requirements of a ligand binding site on

estrogen receptors. Further investigations are currently underway to better understand these descriptors since receptor-based mechanisms are thought to be important in the etiology of estrogen-induced cancer.

The projected potency of metabolites that contain the 6.0 Å descriptor in the mouse CPDB is 52 CASE units for both TMX and TRM metabolites. This value translates into a TD_{50} value of 0.28 mmol/kg per day. The projected potency of metabolites that contain the 8.4 Å descriptor in the rat CPDB is 85 CASE units for both TMX and TRM metabolites that translates into approx. 0.01 mmol/kg per day.

4. Discussion

While TMX, TRM and the majority of their metabolites are predicted to be non-mutagenic in *Salmonella*, the possibility exists that metabolism of the putative desmethyl intermediates may yield hydroxylamines capable of modifying the deoxycytidine and deoxyguanosine moieties in DNA. Thus TMX and TRM are potentially genotoxic. This is supported by the finding that the major DNA-adduct in TMX-treated rats involves deoxyguanosine (Martin et al., 1995).

In the two rodent databases TMX, TRM and their metabolites are predicted to be carcinogenic with an indication that metabolism is required to achieve the full carcinogenic potential, i.e., metabolites of both have higher projected potencies and broader spectra of activity than the parent compounds.

Both TMX and TRM gain carcinogenic attributes associated with the mouse and rat CPDB. These observations suggest that metabolism is likely an important step in the carcinogenic potentiation of these two chemicals. In fact, 4-hydroxy-TMX has a higher binding affinity with the estrogen receptor than does TMX (reviewed by Lerner and Jordan, 1990). For both the rat and mouse CPDB, hydroxylation at the *para* position is a requirement for the predicted carcinogenicity.

Although we found evidence that TMX has the potential for metabolism to a mutagenic hydroxylamine the preponderance of evidence from this investigation suggests that both TMX and TRM both may be carcinogenic by an alternate mechanism. *p*-Hydroxy metabolites of both TMX and TRM are predicted to be carcinogenic in the rat and mouse CPDB. In both instances this is due to distance descriptors. These biophores may be indicative of a receptor-based mechanism of estrogen-induced carcinogenesis.

Our present analyses suggest that TRM is a rodent carcinogen. This is not in accord with results reported heretofore. It should be noted, however, that the carcinogenicity of this antiestrogen has not been investigated systematically to rule out this possibility.

Acknowledgements

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Structural analysis of a group of phytoestrogens for the presence of a 2-D geometric descriptor associated with non-genotoxic carcinogens and some estrogens

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Abstract

Analysis of a group of phytoestrogens indicates that the majority of these chemicals are devoid of a 6Å distance descriptor biophore. This 6Å biophore is associated with the carcinogenicity of a set of chemicals in the mouse subset of the Carcinogenic Potency Database assembled by Gold et al. The prevalence of non-DNA-reactive carcinogens and chemicals endowed with estrogenic activity included in the group of chemicals possessing 6Å descriptor suggests that it describes a ligand binding site on an estrogen receptor. Evidence is presented that estrogens with and without the 6Å biophore bind to alternate receptors or to similar receptors but with different affinities. Since the 6Å biophore was identified based upon a carcinogenicity database, it is conceivable that binding to the putative receptor that recognizes this biophore is associated with carcinogenicity. Alternatively, estrogens devoid of this 6Å biophore may be non-carcinogenic, suggesting that carcinogenicity and estrogenicity may be separate phenomena.

Introduction

Phytoestrogens represent a diverse and naturally occurring source of estrogen exposure in animals and humans. Xenoestrogen exposure has been implicated in the etiology of breast cancer (1) as well as other hormonally-related cancers. Alternatively, dietary intake of certain phytoestrogens has been suggested as diminishing the risk of certain cancers by their possible antiestrogenic action (see 2). The distinction between estrogen and antiestrogen may in fact be obscure. Several of the factors that determine the estrogenicity *vs* antiestrogenicity of a chemical are species- and tissue-specificity, and dose (reviewed in 3). Thus a debate exists over the possible risks and benefits from phytoestrogen exposure.

Traditional structure-activity relationship (SAR) analyses related to cancer causation have been based upon the somatic mutation and electrophilic theory of cancer causation (4). Detailed analyses of large cancer bioassay databases by Ashby and colleagues (5, 6) demonstrate the successful utilization of the electrophilic theory in identifying chemical substructures that are implicated in "genotoxic" carcinogenesis.

In fact, the majority of recognized human carcinogens are genotoxic (7; 8; 9). An exception to this generalization is found with hormonal carcinogens. Hormonal carcinogens are thought to act by a receptor-mediated mechanism (10; 11). However, some evidence also exists for the DNA-reactivity of diethylstilbestrol (12; 13) and tamoxifen (14; 15, 16, 17). This apparent multiplicity of action of certain hormonal carcinogens (i.e., estrogenic and genotoxic) must be considered when examining the possible risks (or benefits) of exposure to phytoestrogens.

The suitability of SAR and quantitative SAR (QSAR) involving non-genotoxic carcinogens is a matter of debate. The multiplicity of mechanisms believed to be involved in non-genotoxic carcinogenesis, has been seen as making such an endeavor problematic (18, 19, 20, however see also 21). However, in a recent study, we identified a 6Å geometric distance descriptor that appears to be associated with non-genotoxic carcinogens and may, in fact, be indicative of a ligand binding site for an estrogen receptor (22, 23). Additionally, estrogens with and without the 6Å biophore may bind to alternate receptors or with different affinities to the same receptors. Of course the possession of this biophore is only indicative of carcinogenicity and possible estrogenicity. Lipophilicity, steric constraints as well as other physico-chemical parameters will influence the binding ability and activities of these chemicals. The results contain herein represent excerpts and extensions of our work (22, 23) to phytoestrogens.

Methods

The computer automated structure evaluation system MULTICASE (MC) (24, 25) was used for these analyses. Basically, the system selects its own descriptors automatically from a learning set of chemicals composed of active and inactive molecules. The descriptors are continuous fragments that are embedded in the molecule. It is assumed that each fragment is not related to activity and will be randomly distributed between inactive and active molecules. Fragments that deviate from a random distribution are considered relevant to activity. The descriptors consist of either activating or inactivating fragments or geometric distance descriptors based on molecular lipophilic centers (see below). MC then selects the most important of these descriptors as a biophore, *i.e.*, the functionality that is associated with

the largest number of active molecules and fewest numbers of inactive molecules. These molecules then become a learning subset to identify the chemical and physico-chemical properties associated with their activity. This will result in a QSAR equation for this group of molecules. The molecules explained here are removed from the set and the process is iterated until all molecules are explained. Once the training set has been assimilated, MC can be queried regarding the predicted activity and potency of molecules of unknown activity.

MC incorporates the following rules to identify two-dimensional distance descriptors based upon the presence of lipophilic centers. These two-dimensional distances are calculated from the molecular structure. Heteroatoms and lipophilic carbon atoms are designated as "special" atoms. A carbon atom is designated as a lipophilic center if it is at least four bonds away from a heteroatom and is also the furthest carbon from the heteroatom when its neighbors are considered. After all the "special" atoms are selected the distances between all possible pairings is calculated. The distribution of these descriptors among active and inactive molecules is analyzed for statistical significance. Various atom groupings are also investigated (i.e. hydrogen bond acceptors and donors as well as halogens).

The learning set used in this investigation was the Carcinogenic Potency Database (CPDB) assembled by Gold et al. (26, 27, 28, 29, 30). A mouse database was extracted from this compilation. The rationale for using a species-specific database as opposed to the entire CPDB is that a more coherent structural descripotrs of carcinogenic activity may be expected in a more strictly defined system.

All dosages for chemicals reported were transformed into gavage equivalents. A carcinogenic potency value (TD_{50}) was calculated for each chemical. The calculated TD_{50} is the dose required for 50% of the animals to remain cancer free throughout the course of the experiment (thus accounting for spontaneous cancer) (26, 31). Additionally, the reported TD_{50} value in mg/kg/day was converted into mmol/kg/day. For the purpose of the SAR analyses, TD_{50} values were transformed into CASE activity units using equation 1.

Chemicals were also assigned to activity groups. Chemicals reported by the authors to be non-carcinogenic in mice were assigned 10 CASE units together with chemicals exhibiting TD_{50} values in excess of 51 mmol/kg/day. The chemicals in the range of 10 to 19 CASE units are designated as inactive. Chemicals with activities in the range of 20 to 29 CASE units are designated marginally active carcinogens and chemicals in the range of 30 to 99 CASE units are considered carcinogenic. Overall, the mouse CPDB consists of 636 chemicals, 291 of which are active, 11 are marginal and 334 are non-carcinogenic.

$$\text{CASE activity} = 14.133 * \log (1 / TD_{50}) + 44.133 \qquad \text{(Equation 1)}$$

Results and discussion

The 6Å biophore was identified as being associated with carcinogenicity in the mouse CPDB. Among the chemicals in the database that possessed the 6Å biophore were estradiol and related chemicals (Figure 1), suggesting that this biophore may be related to the estrogenicity as well as the carcinogenicity of chemicals that contain it (21). In our original study a series of 42 chemicals reported to be endowed with estrogenic activity were tested for the presence of the 6Å biophore; of these about one-half contained the 6Å distance descriptor (see Table 1 for an abbreviated list).

The therapeutic antiestrogen tamoxifen lacked the 6Å biophore; however its active antiestrogenic metabolite, 4-hydroxytamoxifen possessed it. Oxidative metabolism is often required for chemicals to gain their estrogenicity as is the case for tamoxifen (3). Polychlorinated biphenyls and methoxychlor have also been shown to require oxidative metabolism to exhibit estrogenicity (32), and in fact hydroxylated metabolites of polychlorinated biphenyls display the 6Å descriptor.. Moreover, the antiestrogens ICI 164,384 and ICI 182,780 contain the 6Å biophore while LY 117018, another antiestrogen, lacks it. It has been suggested that LY 117018 and tamoxifen (32, 34) and LY 117018 and ICI 182,780 and 164,384 (35) have different bases for their antiestrogenicity. Thus possession or lack of the 6Å biophore may be indicative of a dichotomy that exists among estrogens.

The present exercise entailed analysis for the 6Å biophore in a group of 54 phytoestrogens (Table 1). As mentioned above, oxidative metabolism may be needed to transform estrogens (i.e., proestrogens) into active congeners. Many of the chemicals in this set were hydroxylated and methoxylated congeners of flavonoids. Thus if oxidative metabolism was involved in the conversion of these chemicals to carcinogens or estrogens, this set of chemicals may contain active congeners. However, only indenestrol A and 4,4'-dihydroxystilbene, an analog of diethylstilbestrol, possessed the 6Å descriptor (Table 1). No flavonoids or other phytoestrogens tested contained the 6Å biophore.

Conclusions

We postulated that the 6Å descriptor is associated not only with murine carcinogenicity, but also with an estrogen-receptor ligand. This suggests that the phenomena of estrogenicity and carcinogenicity may result from a similar mechanism as is possibly the case for the reported carcinogenicity of tamoxifen. However, a large population of chemicals endowed with estrogenic activity, particularly phytoestrogens, are devoid of this biophore, suggesting that a structurally-based dichotomy may exist in the estrogenic response.

In fact Baker (36) suggests that the estrogenicity of phytoestrogens derives from their inherent ability to interact with mammalian enzymes involved in the regulation and production of endogenous estrogens. Thus phytoestrogens may exert their estrogenicity through a mechanism that is not involved with carcinogenicity as indicated by the absence of the 6Å descriptor associated with carcinogenicity in mice.

Acknowledgments

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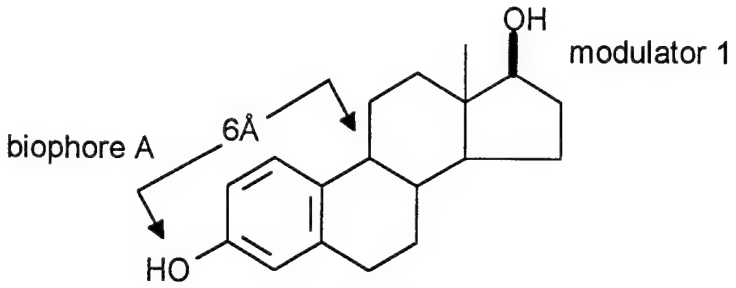
Biophore A:
2D fragment [C-] <--- 6.0 Å ---> [OH-] conjugated and generic

14 out of the known 16 molecules (87%) containing such a biophore are mouse carcinogens with an average activity of 47. (conf.level= 100%)

	Constant	51.8
Modulator 1: OH-CH-	Activating	34.2

The probability that this molecule is a mouse carcinogen is 83.3%

The compound is predicted to be extremly active
The projected mouse carcinogenicity activity 86 CASE units



17β-estradiol

Figure 1. Prediction of the murine carcinogenicity of 17β-estradiol. An activity of 86 CASE units indicates a TD_{50} value of 0.001 mmol/kg/day.

Table 1. Distribution of the 6Å distance descriptor biophore among estrogenic chemicals.

chemical	type	6Å
chalcone	phytoestrogen	-
4-hydroxychalcone	phytoestrogen	-
4,4'-dihydroxychalcone	phytoestrogen	-
4-hydroxy-4'-methoxychalcone	phytoestrogen	-
2',4,4'-trihydroxychalcone (isoliquiritigenin)	phytoestrogen	-
2',4,4'6'-tetrahydroxychalcone (naringenin chalcone)	phytoestrogen	-
2',4,4',6'-tetrahydroxydihydrochalcone (phloretin)	phytoestrogen	-
flavone	phytoestrogen	-
3-hydroxyflavone	phytoestrogen	-
4'-hydroxyflavone	phytoestrogen	-
6-hydroxyflavone	phytoestrogen	-
7-hydroxyflavone	phytoestrogen	-
3',6-dihydroxyflavone	phytoestrogen	-
3',7-dihydroxyflavone	phytoestrogen	-
4',5-dihydroxyflavone	phytoestrogen	-
4',6-dihydroxyflavone	phytoestrogen	-
5,7-dihydroxyflavone (chrysin)	phytoestrogen	-
7,8-dihydroxyflavone	phytoestrogen	-
3',4',7-trihydroxyflavone	phytoestrogen	-
3,5,7- rihydroxyflavone (galangin)	phytoestrogen	-
4',5,7-trihydroxyflavone (apigenin)	phytoestrogen	-
4',7,8-trihydroxyflavone	phytoestrogen	-
3,3',4',7-tetrahydroxyflavone (fisetin)	phytoestrogen	-
3',4',5,7-tetrahydroxyflavone (luteolin	phytoestrogen	-
3,4',5,7-tetrahydroxyflavone (kaempferol)	phytoestrogen	-
3,5,7-trihydroxy-4'-methoxyflavone (kaempferide)	phytoestrogen	-
3,3',4',5,7-pentahydroxyflavone (quercetin)	phytoestrogen	-
2',3,4',5,7-pentahydroxyflavone (morin)	phytoestrogen	-
3,3',4',5,5',7-hexahydroxyflavone (myricetin)	phytoestrogen	-
flavanone	phytoestrogen	-
4',7-dihydroxyflavanone	phytoestrogen	-
4',5,7-trihydroxyflavanone (naringenin)	phytoestrogen	-
3',5,7-trihydroxy-4'-methoxyflavanone (hesperetin)	phytoestrogen	-
3,3',4',5,7-pentahydroxyflavanone (taxifolin)	phytoestrogen	-
3,3',4',5,7,-flavan pentol flavanone ([+/-] catechin)	phytoestrogen	-
isoflavone	phytoestrogen	-
4',7-dihydroxyisoflavone (diadzein)	phytoestrogen	-
7-hydroxy-4'-methoxyisoflavone (formononetin)	phytoestrogen	-
3',4',7-trihydroxyisoflavone	phytoestrogen	-
4',5,7,trihydroxyisoflavone (genistein)	phytoestrogen	-
4',6,7-trihydroxyisoflavone	phytoestrogen	-
5,7-dihydroxy-4'-methoxyisoflavone (biochanin A)	phytoestrogen	-
coumestrol	phytoestrogen	-
4,4'-dihydroxystilbene	phytoestrogen	+
3,5-dihydroxystilbene	phytoestrogen	-
coumarin	phytoestrogen	-
α-sitosterol	phytoestrogen	-
β-sitosterol	phytoestrogen	-
glucyrrhetinic acid	phytoestrogen	-
zearalenol	phytoestrogen	-
zearalenone	phytoestrogen	-
α-zearalenol	phytoestrogen	-

Table 1 continued. Distribution of 6Å distance descriptor biophore among estrogenic chemicals.

chemical	type	6Å
indenestrol A	phytoestrogen	+
tetrahydrocannabinol	phytoestrogen	-
o,p'-DDE	xenoestrogen	-
chlordecone	xenoestrogen	-
diethylstilbestrol	estrogen	+
3'-hydroxy-E-diethylstilbestrol	estrogen	+
4',4"-diethylstilbestrol quinone	estrogen	-
tamoxifen	antiestrogen	-
3-hydroxytamoxifen	antiestrogen	-
4-hydroxytamoxifen acid	antiestrogen	+
toremifene	antiestrogen	-
4-hydroxy-deamino-hydroxytoremifene	antiestrogen	+
ICI 164,384	antiestrogen	+
ICI 182,780	antiestrogen	+
LY 117018	antiestrogen	-
MER 25	antiestrogen	-
17β-estradiol	estrogen	+
17α-ethinyl estradiol	estrogen	+
benzestrol	estrogen	+
dienestrol	estrogen	+
estriol	estrogen	+
estrone	estrogen	+
hexestrol	estrogen	+
megestrol	estrogen	-
norgestrol	estrogen	-
norlestrin (isomer)	estrogen	+
phenol red	xenoestrogen	-

(A)

Communication

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Disruption of Oncogenic K-Ras4B Processing and Signaling by a Potent Geranylgeranyltransferase I Inhibitor*

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Prenylation of the carboxyl-terminal *CAAX* (C, cysteine; *A*, aliphatic acid; and *X*, any amino acid) of Ras is required for its biological activity. We have designed a *CAAX* peptidomimetic, GGTI-287, which is 10 times more potent toward inhibiting geranylgeranyltransferase I (GGTase I) *in vitro* (IC_{50} = 5 nM) than our previously reported farnesyltransferase inhibitor, FTI-276. In whole cells, the methyl ester derivative of GGTI-287, GGTI-286, was 25-fold more potent (IC_{50} = 2 μM) than the corresponding methyl ester of FTI-276, FTI-277, toward inhibiting the processing of the geranylgeranylated protein Rap1A. Furthermore, GGTI-286 is highly selective for geranylgeranylation over farnesylation since it inhibited the processing of farnesylated H-Ras only at much higher concentrations (IC_{50} > 30 μM). While the processing of H-Ras was very sensitive to inhibition by FTI-277 (IC_{50} = 100 nM), that of K-Ras4B was highly resistant (IC_{50} = 10 μM). In contrast, we found the processing of K-Ras4B to be much more sensitive to GGTI-286 (IC_{50} = 2 μM). Furthermore, oncogenic K-Ras4B stimulation of mitogen-activated protein (MAP) kinase was inhibited potently by GGTI-286 (IC_{50} = 1 μM) but weakly by FTI-277 (IC_{50} = 30 μM). Significant inhibition of oncogenic K-Ras4B stimulation of MAP kinase by GGTI-286 occurred at concentrations (1–3 μM) that did not inhibit oncogenic H-Ras stimulation of MAP kinase. The data presented in this study provide the first demonstration of selective disruption of oncogenic K-Ras4B processing and signaling by a *CAAX* peptidomimetic. The higher sensitivity of K-Ras4B toward a GGTase I inhibitor has a tremendous impact on future research directions targeting Ras in anticancer therapy.

Ras is a small guanine nucleotide binding protein that cycles between its active (GTP-bound) and inactive (GDP-bound) forms to transduce growth and differentiation signals from receptor tyrosine kinases to the nucleus (1, 2). Binding of epidermal and platelet-derived growth factors to their receptor tyrosine kinases results in autophosphorylation and recruitment of key signaling proteins to the receptor. Among these proteins are the Ras exchange factors that activate Ras by catalyzing the exchange of GDP for GTP. GTP-bound Ras activates a cascade of mitogen-activated protein (MAP)[1] kinases by recruiting Raf to the plasma membrane. Raf, a serine/threonine kinase, phosphorylates MAP kinase kinase, which in turn activates MAP kinase by phosphorylating it on threonine and tyrosine. Hyperphosphorylated MAP kinase translocates to the nucleus where it phosphorylates transcription factors that are involved in the regulation of growth-related genes. The growth signal is terminated when Ras hydrolyzes GTP to GDP (1–3). However, mutations that lock Ras in its GTP-bound form result in an uninterrupted growth signal and are believed to contribute to the development of more than one-third of human cancers (4, 5).

In order for Ras to transduce its normal and oncogenic signal it must be anchored to the plasma membrane, which is accomplished by post-translational modifications that increase its hydrophobicity (6–8). A key step in this process is catalyzed by farnesyltransferase (FTase), an enzyme that transfers farnesyl from farnesylpyrophosphate, a cholesterol biosynthesis intermediate, to the cysteine of the carboxyl-terminal *CAAX* of Ras (C, cysteine, *A*, aliphatic amino acid; *X*, serine or threonine) (9, 10). A closely related enzyme, geranylgeranyltransferase I (GGTase I), attaches the lipid geranylgeranyl to the cysteine of the *CAAX* box of proteins, where *X* is leucine (11, 12). FTase and GGTase I are α/β heterodimers that share the α subunit (13, 14). Cross-linking experiments suggested that both substrates (farnesylpyrophosphate and Ras *CAAX*) interact with the β subunit of FTase (15, 16). Although GGTase I prefers leucine at the *X* position, its substrate specificity was shown to overlap with that of FTase *in vitro* (17). Furthermore, GGTase I was also able to transfer farnesyl to a leucine terminating peptide (18).

Because farnesylation of Ras is required for its oncogenic activity, we (19–22) and others (23–27) have designed potent inhibitors of FTase as potential anticancer drugs. These inhibitors are *CAAX* peptidomimetics, which show great selectivity for FTase over GGTase I *in vitro* and selectively block the processing of farnesylated but not geranylgeranylated proteins in whole cells (22). Furthermore, FTase inhibitors can selectively block oncogenic Ras signaling and reverse malignant phenotype at concentrations that do not affect normal cells (24, 25). However, mammalian cells express four types of Ras proteins (H-, N-, KA-, and KB-Ras) among which K-Ras4B is the most frequently mutated form of Ras in human cancers (4, 5). Although several laboratories have demonstrated potent inhibition of oncogenic H-Ras processing and signaling (26, 28), this disruption has not been shown with K-Ras4B. Hence, a drawback of the previous studies is the use of H-Ras and not

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‖ Recipient of a predoctoral fellowship from the Andrew Mellon Foundation.

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[1] The abbreviations used are: MAP, mitogen-activated protein; FTase, farnesyltransferase; GGTase I, geranylgeranyltransferase I; PAGE, polyacrylamide gel electrophoresis; *CAAX*, tetrapeptide where C is cysteine, *A* is aliphatic amino acid, and *X* is serine or methionine; FTI, farnesyltransferase inhibitor; GGTI, geranylgeranyltransferase inhibitor.

K-Ras4B as a target for the development of these inhibitors. Recently, we have shown that a potent inhibitor of FTase disrupts K-Ras4B processing but only at very high concentrations that also inhibited the processing of geranylgeranylated proteins (29). This suggested that K-Ras4B may be geranylgeranylated, particularly in cells where FTase is inhibited. Consistent with this possibility is the recent observation that K-Ras4B can be geranylgeranylated *in vitro*, but its K_m for GGTase I is 7 times higher than its K_m for FTase (30). GGTase I *CAAX*-based inhibitors that can block geranylgeranylation processing have not been reported. In the present study, we have designed a *CAAX* peptidomimetic that selectively inhibits GGTase I and demonstrate that oncogenic K-Ras4B processing and signaling are disrupted at concentrations that affect geranylgeranylation but not farnesylation processing.

EXPERIMENTAL PROCEDURES

Synthesis of FTase and GGTase I Inhibitors—Peptidomimetics FTI-276 and FTI-277 were prepared as described previously (29). The GGTase I inhibitors GGTI-287 and -286 were prepared from 2-phenyl-4-nitrobenzoic acid (29) by reaction with L-leucine methyl ester followed by reduction with stannous chloride. The resulting 4-amino-2-phenylbenzoyl leucine methyl ester was reacted with *N-t*-butoxycarbonyl-*S*-trityl-cysteinal and deprotected by procedures similar to those described for the FTase inhibitors (29) to give GGTI-286 and -287 as their hydrochloride salts.

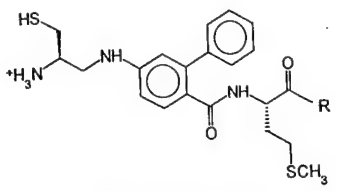
FTase and GGTase I Activity Assay—FTase and GGTase I activities from 60,000 × *g* supernatants of human Burkitt lymphoma (Daudi) cells (ATCC, Rockville, MD) were assayed exactly as described previously for FTase (22). Inhibition studies were performed by determining the ability of Ras *CAAX* peptidomimetics to inhibit the transfer of [^{3}H]farnesyl and [^{3}H]geranylgeranyl from [^{3}H]farnesylpyrophosphate and [^{3}H]geranylgeranylpyrophosphate to H-Ras-CVLS and H-Ras-CVLL, respectively (22).

Ras and Rap1A Processing Assay—H-Ras cells (31) and K-Ras4B cells (32) were kind gifts from Dr. Channing Der and Dr. Adrienne Cox (University of North Carolina, Chapel Hill). Cells were seeded on day 0 in 100-mm dishes in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 1% penicillin/streptomycin. On days 1 and 2, cells were refed with medium containing various concentrations of FTI-277, GGTI-286, or vehicle (10 mM dithiothreitol in dimethyl sulfoxide). On day 3, cells were washed and lysed in lysis buffer containing 50 mM HEPES, pH 7.5, 10 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM $MgCl_2$, 1 mM EGTA, 25 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 2 mM Na_3V0_4, 1 mg/ml soybean trypsin inhibitor. 10 μg/ml aprotinin, 6.4 mg/ml Sigma-104® phosphatase substrate. Lysates were cleared (14,000 rpm, 4 °C, 15 min), and equal amounts of protein were separated on a 12.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted using an anti-Ras antibody (Y13–259, ATCC) or an anti-Rap1A antibody (SC-65, Santa Cruz Biotechnology, Santa Cruz, CA). Antibody reactions were visualized using either peroxidase-conjugated goat anti-rat IgG (for Y13–259) or peroxidase-conjugated goat anti-rabbit IgG (for Rap1A) and an enhanced chemiluminescence detection system (ECL, Amersham Corp.), as described previously (22).

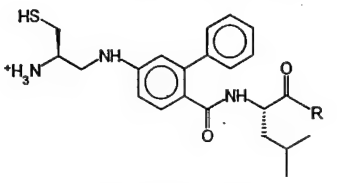
MAP Kinase Immunoblotting—Cells were treated with FTI-277, GGTI-286, or vehicle and lysed as described previously for Ras and Rap1A processing. Equal amounts of protein were separated on 15% SDS-PAGE, transferred to nitrocellulose, and immunoblotted using an anti-MAP kinase antibody (Erk2, monoclonal, UBI, Lake Placid, NY). Antibody reactions were visualized using peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and an enhanced chemiluminescence detection system (ECL, Amersham Corp.).

RESULTS AND DISCUSSION

The carboxyl-terminal *CAAX* tetrapeptide of Ras is a substrate for FTase and serves as a target for designing inhibitors of this enzyme with potential anticancer activity (23). We have recently made a highly potent (IC_{50} = 500 pM) inhibitor of FTase, FTI-276 (Fig. 1) (29). Its cell-permeable methyl ester FTI-277 inhibited H-Ras processing in whole cells with an IC_{50} of 100 nM (29). Furthermore, FTI-276 is highly selective (100-fold) for FTase over GGTase I (Table I). Although oncogenic H-Ras processing and signaling were exquisitely sensitive to



FTI-276: R = O⁻
FTI-277: R = OCH_3



GGTI-286: R = OCH_3
GGTI-287: R = O⁻

FIG. 1. ***CAAX* peptidomimetic structures.** Structures of FTI-276/277 and GGTI-287/286 are shown.

TABLE I
In vitro and in vivo inhibition of GGTase I and FTase

	In vitro (IC_{50})			*In vivo* processing (IC_{50})		
	FTase	GGTase I		H-Ras	K-Ras	Rap1A
	nM				μM	
FTI-276	0.5	50	FTI-277	0.1	10	50
GGTI-287	25	5	GGTI-286	>30	2	2

FTI-277, those of K-Ras4B were highly resistant. However, at high concentrations of FTI-277, when the processing of the geranylgeranylated Rap1A protein was inhibited, K-Ras4B processing was also inhibited (29). We, therefore, set out to determine whether a GGTase I-selective inhibitor would disrupt K-Ras4B processing and signaling. Our approach involved replacing the central "*AA*" of *CAAX* tetrapeptides by a hydrophobic spacer and incorporating a leucine residue in the carboxyl-terminal position to optimize recognition by GGTase I. We herein report a CAAL peptidomimetic, GGTI-287, where reduced cysteine is linked to leucine by 2-phenyl-4-aminobenzoic acid (Fig. 1). The phenyl substituent was designed to occupy the hydrophobic dipeptide *AA* binding pocket that must be present in the enzyme. GGTI-287 potently inhibited GGTase I *in vitro* (IC_{50} = 5 nM) and was selective toward inhibiting GGTase I over FTase (IC_{50} = 25 nM) (Table I). Thus, the substitution of methionine in FTI-276 by a leucine in GGTI-287 (Fig. 1) increased the potency toward GGTase I by approximately 10-fold (Table I). More importantly, it reversed the selectivity from a FTase to a GGTase I-specific inhibitor by a factor of 500 (Table I). To determine whether this selectivity is respected in whole cells, we have synthesized the cell-permeable methyl ester derivative of GGTI-287, GGTI-286 (Fig. 1), and treated NIH 3T3 cells, which overexpress oncogenic H-Ras-CVLS (31), with GGTI-286 (0–30 μM). Cell lysates were electrophoresed on SDS-PAGE and immunoblotted with an anti-Ras antibody as described under "Experimental Procedures." Fig. 2 shows that accumulation of unprocessed H-Ras did not occur until 30 μM GGTI-286. Therefore, GGTI-286 is not a good inhibitor of H-Ras processing in whole cells. However, GGTI-286 was a very potent inhibitor of the processing of the geranylgeranylated Rap1A protein (IC_{50} = 2 μM) (Fig. 2). Thus, GGTI-286 is more than 15-fold selective for inhibition of geranylgeranylation over farnesylation processing (Table I). These data are in direct contrast to the FTase-specific inhibitor FTI-277, which inhibited H-Ras and Rap1A processing with IC_{50}s of 100 nM and 50 μM, respectively (Fig. 2). Thus, GGTI-286 is

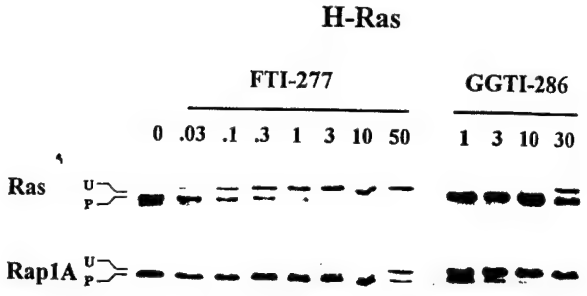


FIG. 2. **Disruption of H-Ras and Rap1A processing.** NIH 3T3 cells that overexpress oncogenic H-Ras were treated with various concentrations of FTI-277 (0–50 μM) or GGTI-286 (0–30 μM). The cells were lysed, and the lysates were electrophoresed on SDS-PAGE and immunoblotted with either anti-Ras or anti-Rap1A antibodies as described under "Experimental Procedures." *U* and *P* designate unprocessed and processed forms of the proteins. Data are representative of three independent experiments.

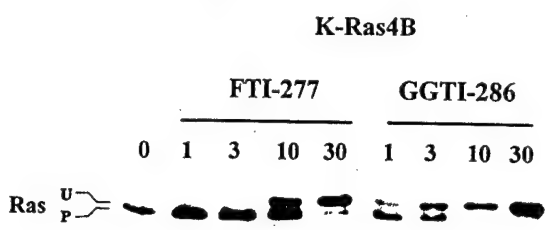


FIG. 3. **Disruption of K-Ras4B processing.** NIH 3T3 cells that overexpress oncogenic K-Ras4B were treated with FTI-277 or GGTI-286 (0–30 μM). The cells were lysed and the lysates were electrophoresed on SDS-PAGE and immunoblotted with anti-Ras antibodies as described under "Experimental Procedures." *U* and *P* designate unprocessed and processed forms of Ras. The data are representative of three independent experiments.

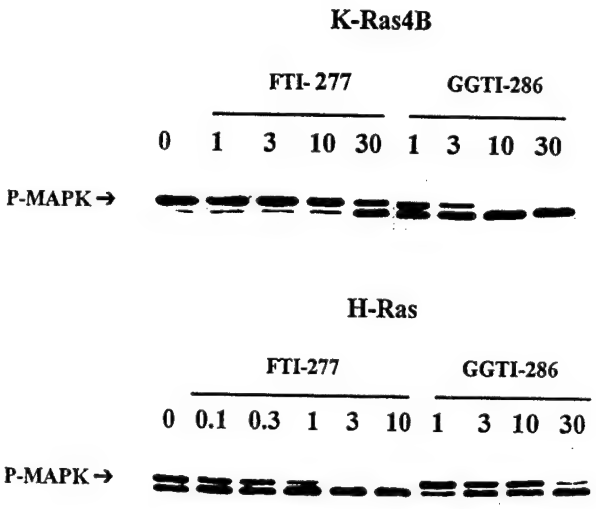


FIG. 4. **Inhibition of oncogenic activation of MAP kinase.** NIH 3T3 cells that overexpress either oncogenic H-Ras or K-Ras4B were treated with either FTI-277 or GGTI-286 (0–30 μM). The cells were lysed, and the lysates were electrophoresed on SDS-PAGE and immunoblotted with an anti-MAP kinase antibody. *P-MAPK* designates hyperphosphorylated MAP kinase. The data are representative of three independent experiments.

25-fold more potent than FTI-277 at inhibiting geranylgeranylation in whole cells (Table I).

We next evaluated the ability of GGTI-286 to inhibit the processing and signaling of oncogenic K-Ras4B. NIH 3T3 cells, which overexpress oncogenic K-Ras4B (32), were treated with either GGTI-286 (0–30 μM) or FTI-277 (0–30 μM), and the lysates were immunoblotted with an anti-Ras antibody as described under "Experimental Procedures." Fig. 3 shows that GGTI-286 inhibited potently the processing of K-Ras4B with an IC_{50} of 2 μM. The ability of GGTI-286 to inhibit the processing of K-Ras4B was much closer to its ability to inhibit the processing of geranylgeranylated Rap1A (IC_{50} = 2 μM) than that of farnesylated H-Ras (IC_{50} > 30 μM) (Fig. 2 and Table I). This suggested that K-Ras4B might be geranylgeranylated. Consistent with this is the fact that K-Ras4B processing was very resistant to the FTase-specific inhibitor FTI-277 (IC_{50} = 10 μM) (Fig. 3). Furthermore, GGTI-286 inhibited K-Ras4B processing at concentrations (1–3 μM) (Fig. 3) that had no effect on the processing of farnesylated H-Ras (Fig. 2). These results are not consistent with the work of Casey *et al.* (7), who used [^{3}H]mevalonic acid to label cellular proteins and provided evidence for a farnesylated K-Ras4B based on high pressure liquid chromatography of the radiolabeled prenyl group. However, the mass of the prenyl group in these studies was not determined.

To determine whether inhibition of K-Ras4B processing by GGTI-286 results in disruption of oncogenic signaling, we evaluated the ability of GGTI-286 to antagonize oncogenic K-Ras4B constitutive activation of MAP kinase. Activated MAP kinase is hyperphosphorylated and migrates slower than hypophosphorylated (inactive) MAP kinase on SDS-PAGE (26, 29). Fig. 4 shows that K-Ras4B-transformed cells contained mainly activated MAP kinase. Treatment of these cells with the FTase-specific inhibitor FTI-277 (0–30 μM) did not inhibit MAP kinase activation until 30 μM (Fig. 4). In contrast, GGTI-286 inhibited MAP kinase activation with an IC_{50} of 1 μM, and the block was complete at 10 μM. Thus, GGTI-286 blocked oncogenic K-Ras4B MAP kinase activation at a concentration (10 μM) where FTI-277 had no effect. In contrast, oncogenic H-Ras activation of MAP kinase was inhibited only slightly by GGTI-286 whereas FTI-277 completely blocked this activation at 3 μM (Fig. 4). Furthermore, GGTI-286 blocked K-Ras4B activation of MAP kinase at a concentration (10 μM) that had little effect on H-Ras activation of MAP kinase (Fig. 4). It should be noted that GGTI-286 was not toxic to cells at concentrations as high as 10 μM. However, at higher concentrations (30 μM), GGTI-286 did show some signs of toxicity as reflected by a rounded morphology of the cells. Thus, GGTI-286 was not toxic at concentrations (10 μM) that resulted in complete inhibition of MAP kinase activation.

Recently, we have demonstrated that the FTase-specific inhibitor FTI-277 inhibits oncogenic H-Ras processing and signaling (29) and blocks *in vivo* tumor growth of H-Ras-transformed NIH 3T3 cells and a human lung carcinoma that expresses a K-Ras mutation (33). However, processing of K-Ras4B was inhibited by FTI-277 only at high concentrations similar to those needed to inhibit the processing of the geranylgeranylated protein Rap1A (29). In the present study, we have described the design of a geranylgeranylation-specific inhibitor and its effects on oncogenic K-Ras4B processing and signaling. Our results demonstrate that oncogenic K-Ras4B processing and constitutive activation of MAP kinase are potently inhibited by a GGTase I-selective inhibitor (GGTI-286) but are resistant to one selective for FTase (FTI-277). This is in direct contrast to the processing and signaling of oncogenic H-Ras, which was very sensitive to FTI-277 and highly resistant to GGTI-286. The resistance of K-Ras4B to disruption by FTase inhibitors could be explained by the 50-fold higher affinity of K-Ras4B for FTase compared with H-Ras (30). Our current data strongly suggest, however, that K-Ras4B may be resistant to FTase inhibition because it is post-translationally processed by a geranylgeranyl rather than a farnesyl group. This is consistent with the recent observation that *in vitro* K-Ras4B can be geranylgeranylated by GGTase I (30). Al-

though this previous work shows that K-Ras4B is a 7 times better substrate *in vitro* for FTase (K_m = 0.2 μM) than GGTase I (K_m = 1.5 μM) (30), our data suggest that, in cultured cells, K-Ras4B is geranylgeranylated. This is supported by the fact that GGTI-286 inhibited oncogenic K-Ras4B processing and MAP kinase activation at concentrations (1 and 3 μM) that did not affect farnesylation-dependent processing.

The results presented in this study are critical to the further design and development of inhibitors of Ras prenylation as potential anticancer agents. The results identify the GGTase I-specific inhibitor GGTI-286 as a small molecule capable of antagonizing selectively oncogenic K-Ras4B (not H-Ras) signaling. This is a key finding since K-Ras4B is the most frequently identified mutated Ras in human cancers, and its function has been resistant to FTase inhibitors. Furthermore, we have recently shown that a GGTase I inhibitor selectively suppressed activated DRas1 in *Drosophila* without side effects demonstrating the utility of these Ras C*AAX* peptidomimetics in whole animals (34). Finally, the availability of K-Ras4B-selective inhibitors (*i.e.* GGTI-286) in addition to H-Ras-selective inhibitors (*i.e.* FTI-277) will enhance our understanding of the distinctive roles of these two forms of Ras in normal and oncogenic signaling.

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(B)

Ras *CAAX* Peptidomimetic FTI-277 Selectively Blocks Oncogenic Ras Signaling by Inducing Cytoplasmic Accumulation of Inactive Ras-Raf Complexes*

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Ras-induced malignant transformation requires Ras farnesylation, a lipid posttranslational modification catalyzed by farnesyltransferase (FTase). Inhibitors of this enzyme have been shown to block Ras-dependent transformation, but the mechanism by which this occurs remains largely unknown. We have designed FTI-276, a peptide mimetic of the COOH-terminal Cys-Val-Ile-Met of K-Ras4B that inhibited potently FTase *in vitro* (IC_{50} = 500 pM) and was highly selective for FTase over geranylgeranyltransferase I (GGTase I) (IC_{50} = 50 nM). FTI-277, the methyl ester derivative of FTI-276, was extremely potent (IC_{50} = 100 nM) at inhibiting H-Ras, but not the geranylgeranylated Rap1A processing in whole cells. Treatment of H-Ras oncogene-transformed NIH 3T3 cells with FTI-277 blocked recruitment to the plasma membrane and subsequent activation of the serine/threonine kinase c-Raf-1 in cells transformed by farnesylated Ras (H-RasF), but not geranylgeranylated, Ras (H-RasGG). FTI-277 induced accumulation of cytoplasmic non-farnesylated H-Ras that was able to bind Raf and form cytoplasmic Ras/Raf complexes in which Raf kinase was not activated. Furthermore, FTI-277 blocked constitutive activation of mitogen-activated protein kinase (MAPK) in H-RasF, but not H-RasGG, or Raf-transformed cells. FTI-277 also inhibited oncogenic K-Ras4B processing and constitutive activation of MAPK, but the concentrations required were 100-fold higher than those needed for H-Ras inhibition. The results demonstrate that FTI-277 blocks Ras oncogenic signaling by accumulating inactive Ras/Raf complexes in the cytoplasm, hence preventing constitutive activation of the MAPK cascade.

Ras proteins are plasma membrane-associated GTPases that function as relay switches transducing biological information from extracellular signals to the nucleus (for review, see Refs. 1–3). In normal cells, Ras proteins cycle between the GDP (inactive)- and GTP (active)-bound forms to regulate proliferation and differentiation. The mechanism by which extracellular signals, such as epidermal and platelet-derived growth factor, transduce their biological information to the nucleus via Ras proteins has recently been unraveled. Binding of these growth factors to tyrosine kinase receptors results in autophosphorylation of various tyrosines which bind *src*-homology 2 (SH2) domains of several signaling proteins. One of these, a cytoplasmic complex of GRB-2 and a Ras exchanger (SOS-1), is recruited by the tyrosine-phosphorylated receptor, where SOS-1 catalyzes the exchange of GDP for GTP on Ras, hence activating it. GTP-bound Ras recruits c-Raf-1, a serine/threonine kinase, to the plasma membrane where its kinase activity is activated by as yet undetermined membrane-associated events. Raf triggers a kinase cascade by phosphorylating MAP kinase kinase (MEK) which, in turn, phosphorylates MAPK[1] on threonine and tyrosine residues. Activated MAPK translocates to the nucleus where it phosphorylates transcription factors. In a large number of human cancers, Ras is locked in its GTP-bound form due to mutations in amino acids 12, 13, or 61 (4, 5). As a result, the Ras pathway no longer requires an upstream growth signal, is uninterrupted and the enzymes in this pathway such as Raf, MEK, and MAPK are constitutively activated (1–3).

In addition to its inability to hydrolyze GTP, oncogenic Ras must associate with the plasma membrane to cause malignant transformation (6–8). Ras membrane association is mediated through a series of posttranslational modifications (9–12). The first step is catalyzed by a cytosolic heterodimer farnesyltransferase (FTase), which attaches farnesyl to the thiol group of cysteine of the carboxyl-terminal tetrapeptide *CAAX*, where *A* is isoleucine or valine and *X* is serine or methionine (13–16). Because farnesylation is required and sufficient for Ras transformation (8, 17), FTase is an attractive target for the development of a potential new class of anti-cancer agents (18, 19). Although *CAAX* peptides are potent competitive inhibitors of FTase, rapid degradation and low cellular uptake limit their use as therapeutic agents. Over the last 3 years, we (20–22) and others (23–26) have designed *CAAX* peptidomimetics that potently inhibit FTase *in vitro* and Ras processing *in vivo* but that retain several peptidic features. More recently, we have

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; *CAAX*, tetrapeptide where C = cysteine, *A* = aliphatic amino acid, and *X* = serine or methionine; FTase, farnesyltransferase; GGTase I, geranylgeranyltransferase I; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; FTI, farnesyl transferase inhibitor; Boc, *t*-butoxycarbonyl; HPLC, high performance liquid chromatography.

designed non-peptide *CAAX* mimetics that have several desirable features for further development as anti-cancer agents (27). Although these non-peptide mimics and *CAAX* peptidomimetics inhibit FTase potently (nM), their ability to disrupt Ras processing in whole cells occurs at micromolar concentrations that would not be easily achievable in *in vivo* settings. Therefore, there is a need for improved FTase inhibitors with more potent activity in whole cells and *in vivo*.

Ras *CAAX* peptidomimetics have been shown to reverse oncogenic H-Ras transformation, inhibit the growth of H-Ras-transformed, but not normal cells in culture, and slow the growth of Ras but not Raf-transformed cells in nude mice (23, 24, 28). Recently, FTase inhibitors have also been shown to inhibit oncogenic Ras activation of MAPK in H-Ras-transformed cells (29, 30). Whether FTase inhibitors also inhibit oncogenic K-Ras signaling is not yet known. This is an important question, since K-Ras is a more efficient substrate for FTase, rendering it more difficult to block by FTase inhibitors, and since K-Ras mutations are most prevalent in human tumors where Ras is mutated. Furthermore, the mechanism by which FTase inhibitors suppress MAPK activation has not been investigated. Specifically, the effects of FTase inhibitors on the interactions between Ras and its downstream effectors such as Raf have not been studied. The present work describes the design of a highly potent (pM/nM) Ras *CAAX* peptidomimetic which antagonizes both H- and K-Ras oncogenic signaling. The results demonstrate that the mechanism by which this inhibitor blocks Ras-dependent signaling involves sequestering Raf in the cytoplasm away from the plasma membrane where it would be activated.

EXPERIMENTAL PROCEDURES

Synthesis of CAAX Analogues—The peptidomimetic FTI-276 was synthesized as follows: 2-bromo-4-nitrotoluene was coupled with phenylboronic acid. The product was oxidized with $KMnO_4$ to 2-phenyl-4-nitrobenzoic acid which was coupled with L-methionine methyl ester followed by reduction with stannous chloride. The resulting 4-amino-2-phenylbenzoyl methionine methyl ester was reacted with *N*-Boc-*S*-trityl-cysteinal to give *N*-Boc-*S*-trityl methyl ester of FTI-276. This methyl ester was hydrolyzed by LiOH and then deprotected by trifluoroacetic acid. The pure FTI-276 was obtained through preparative HPLC. The peptidomimetic FTI-277 was made from the *N*-Boc-S-trityl methyl ester of FTI-276 by first treatment with mercuric chloride followed by hydrogen sulfide in methanol. The final product, FTI-277, was obtained as its hydrochloride salt. Spectroscopic data of both FTI-276 and FTI-277 were consistent with their assigned structures. HPLC analysis showed purity over 99.9%.

FTase and GGTase I Activity Assays—FTase and GGTase I activities from 60,000 × *g* supernatants of human Burkitt lymphoma (Daudi) cells (ATCC, Rockville, MD) were assayed exactly as described previously (27). Inhibition studies were performed by determining the ability of Ras *CAAX* peptidomimetics to inhibit the transfer of[^{3}H]farnesyl and [^{3}H]geranylgeranyl from [^{3}H]farnesyl pyrophosphate and [^{3}H]geranylgeranyl pyrophosphate to H-Ras-CVLS and H-Ras-CVLL, respectively (27).

Ras and Rap1A Processing Assay—H-RasF, H-RasGG, pZIPneo, Raf, S186 (31), and K-Ras4B cells (17) were seeded on day 0 in 100-mm dishes in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 1% penicillin/streptomycin. On days 1 and 2, cells were refed with medium containing various concentrations of FTI-277 or vehicle (10 mM DTT in Me_2SO). On day 3, cells were lysed in buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 25 μg/ml leupeptin, 1 mM PMSF, 2 mM Na_3VO_4, 1 mg/ml soybean trypsin inhibitor, and 6.4 mg/ml Sigma-104® phosphatase substrate. Lysates were then immunoblotted using an anti-Ras antibody (Y13-238, ATCC) or an anti-Rap1A antibody (SC-65, Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (27).

Co-immunoprecipitation of Raf and Ras—Cells were seeded on day 0 in 100 mM dishes, and on days 1 and 2 cells were treated with FTI-277 (10 μM) or vehicle. On day 3, cells were collected and pellets were resuspended in ice-cold hypotonic buffer (10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF) and sonicated to break up the cell pellet. The cell suspension was then centrifuged at 2,000 rpm for 10 min to clear debris after which the supernatant was spun for 30 min at 100,000 × *g* to separate membrane and cytosol fractions. The cytosol and membrane fractions were then lysed as described previously (32). Equal amounts of cytosol and membrane fractions were immunoprecipitated using 50 μl of a 25% protein A-Sepharose CL-4B suspension (Sigma) with 1 μg/ml anti-c-Raf-1 (SC133, Santa Cruz Biotechnology, Santa Cruz, CA). The samples were washed five times in 50 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 10% glycerol, 20 mM NaF). The final pellets were run on 12.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted for the presence of Ras using anti-Ras antibody (Y13-238) and anti-Raf antibody (SC133, Santa Cruz Biotechnology, Santa Cruz, CA). Detection was the same as above for Ras and Rap1A processing.

Detection of GTP and GDP Bound to Ras—H-RasF cells were seeded and treated as above for Ras/Raf interaction studies. On day 2, however, cells were labeled overnight with [^{32}P]orthophosphate as described previously (32). On day 3, the cytosol and membrane fractions were separated, lysed, and equal amounts of lysate were immunoprecipitated using anti-Ras antibody (Y13-259) along with 30 μl of protein A-agarose goat anti-rat IgG complex (Oncogene Science). Bound nucleotide was eluted and separated by TLC as described previously (32).

Raf-1 Kinase Activity Assay—Raf-1 kinase activity was assayed by determining the ability of Raf to transfer phosphate from [γ-^{32}P]ATP to a 19-mer peptide containing a Raf autophosphorylation site. Membrane and cytosol fraction isolation and Raf immunoprecipitation were described above. Immunoprecipitates were washed with kinase buffer (50 mM Tris, pH 7.3, 150 mM NaCl, 12 mM $MnCl_2$, 1 mM DTT, 0.2% Tween 20). Immune complex kinase assays were performed by incubating immunoprecipitates from membrane and cytosol fractions in 96 μl of kinase buffer with 20 μCi of [γ-^{32}P]ATP (10 mCi/ml, Amersham Corp.) and 2 μl of the Raf-1 substrate peptide IVQQFGFQRRASDDGKLTD (1 mg/ml, Promega, Madison, WI) for 30 min at 25 °C. The phosphorylation reaction was terminated by spotting a 50-μl aliquot onto Whatman P81 phosphocellulose filters. The filters were washed in 0.5% orthophosphoric acid and air-dried. The amount of ^{32}P incorporated was determined by liquid scintillation counting.

MAP Kinase Immunoblotting—Cells were treated with FTI-277 and lysed as described above for Ras and Rap1A processing. Equal amounts of lysate were separated on a 15% SDS-PAGE, transferred to nitrocellulose, and immunoblotted using an anti-MAPK antibody (erk2, monoclonal, Upstate Biotechnology, Inc., Lake Placid, NY). Antibody reactions were visualized using peroxidase-conjugated goat anti-mouse IgG and enhanced chemiluminescence detection (Amersham).

RESULTS AND DISCUSSION

Recently, we (20–22, 27) and others (23–26) have designed Ras *CAAX* peptidomimetics that inhibit FTase potently with concentrations in the nM range. However, these agents inhibited Ras processing in whole cells only at μM levels (29, 30). In order to investigate the mechanism of action of FTase inhibitors, we sought to first improve the potency and selectivity of our first generation of *CAAX* peptidomimetics. Structure activity relationship studies with *CAAX* peptides and peptidomimetics predict a hydrophobic region in the active site of FTase that interacts with the central portion of the *CAAX* tetrapeptide. In our original designs (20–22), we have replaced the central aliphatic dipeptide "VI" in CVIM by aromatic spacers of the aminobenzoic acid family (Fig. 1*A*). Structural comparison of CVIM with the peptidomimetic FTI-249 (Fig. 1*A*) suggests that increased binding energy could be gained by increasing the size and hydrophobicity of the aminobenzoic acid spacer to fully occupy the FTase substrate binding pocket. In the present work, we have designed FTI-276 and its methyl ester FTI-277 (Fig. 1*A*), where reduced cysteine and methionine are linked by 2-phenyl-4-aminobenzoic acid, hence increasing the hydrophobic character of the central portion of the peptidomimetic. FTI-276 and FTI-277 were synthesized as described under "Experimental Procedures." Fig. 1*B* shows that FTI-276 inhibited FTase with an IC_{50} of 500 pM, whereas FTI-249, the unsubstituted precursor to FTI-276, had an IC_{50} of 200,000 pM (27). Thus, a phenyl ring at the 2 position of the aminobenzoic acid spacer increased inhibition potency of FTase by 400-fold, indi-

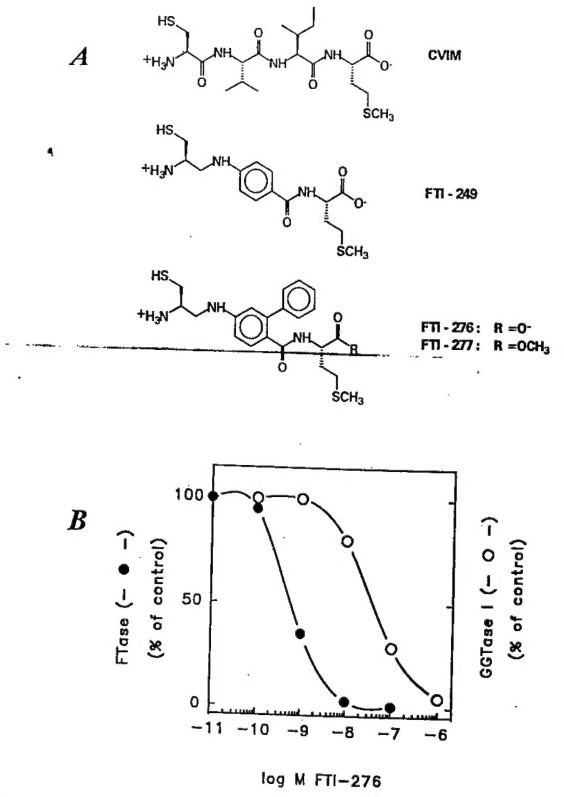

FIG. 1. **Ras *CAAX* peptidomimetics and FTase/GGTase I inhibition.** *A*, structures of CVIM, FTI-249, FTI-276, and FTI-277. *B*, FTase and GGTase I inhibition assays were carried out by determining the ability of FTI-276 to inhibit the transfer of farnesyl and geranylgeranyl to recombinant H-Ras-CVLS and H-Ras-CVLL, respectively. The data are representative of at least three independent experiments.

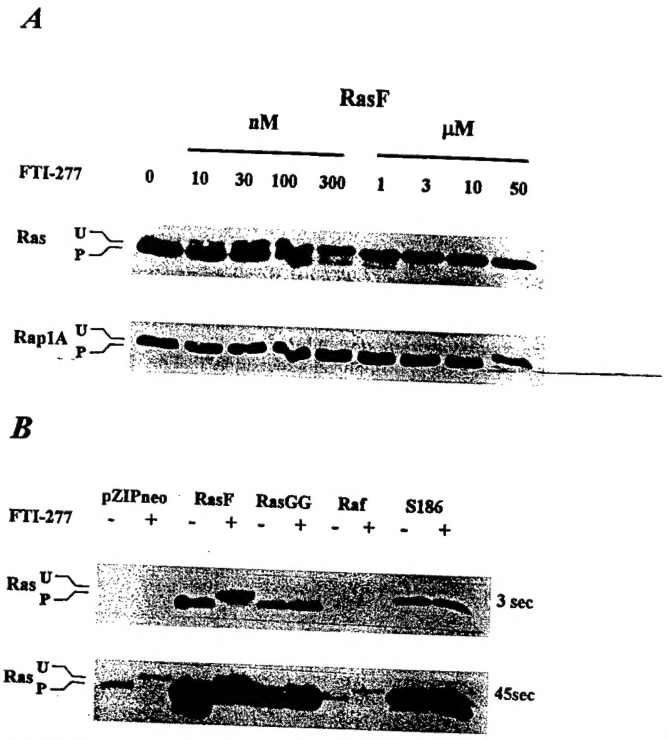

FIG. 2. **Effects of FTI-277 on Ras and Rap1A processing.** *A*, H-RasF cells were treated with various concentrations of FTI-277, lysed, and the lysates were immunoblotted with anti-Ras or anti-Rap1A antibodies as described under "Experimental Procedures." *B*, pZIPneo, H-RasF, H-RasGG, Raf, and S186 cells were treated with vehicle or FTI-277 (10 μM), lysed, and lysates were immunoblotted by anti-Ras antibody. Data are representative of five independent experiments.

cating a significant role for the hydrophobic pocket within the *CAAX* binding site of FTase. This extremely potent inhibitor was also highly selective (100-fold) for FTase over the closely related GGTase I. FTI-276 inhibited GGTase I with an IC_{50} of 50 nM (Fig. 1*B*). This 100-fold selectivity is superior to our previously reported 15-fold selectivity of the parent compound FTI-249 (27). We next determined the ability of FTI-276 to inhibit Ras processing. To facilitate cellular uptake, we have used the corresponding methyl ester, FTI-277 (Fig. 1*A*). H-RasF cells (NIH 3T3 cells transformed with oncogenic (leucine 61) H-Ras-CVLS (31)) were treated with FTI-277 (0–50 μM), and the lysates were blotted with anti-Ras or anti-Rap1A antibodies as described under "Experimental Procedures." Fig. 2*A* shows that concentrations as low as 10 nM inhibited Ras processing but concentrations as high as 10 μM did not inhibit processing of the geranylgeranylated Rap1A (Fig. 2*A*). FTI-277 inhibited Ras processing with an IC_{50} of 100 nM (Fig. 2*A*), whereas the IC_{50} of FTI-249 was 100 μM. Furthermore, the most potent *CAAX* peptidomimetics previously reported inhibited Ras processing in whole cells at micromolar concentrations (28–30). The selectivity of FTI-277 for farnesylation over geranylgeranylation processing in whole cells was further investigated by treating H-RasGG cells (NIH 3T3 cells transformed with oncogenic (leucine 61) H-Ras-CVLL (31)) with FTI-277. Fig. 2*B* shows that the processing of H-RasGG was not affected, whereas that of H-RasF was completely blocked. Furthermore, the processing of endogenous Ras was also blocked in pZIPneo cells (NIH 3T3 cells transfected with empty vector) and Raf cells (NIH 3T3 cells transfected with a transforming mutant of human Raf-1 (Raf22W) (33)). Thus, FTI-277 is a farnesylation-specific inhibitor which blocks the processing of both oncogenic and normal Ras.

In order to determine the mechanism by which FTI-277 disrupts Ras oncogenic signaling, we transfected NIH 3T3 cells with activated (GTP-locked) Ras and first investigated the effects of FTI-277 on the interaction of Ras with its immediate effector c-Raf-1 (1–3, 32). Various NIH 3T3 cell transfectants (pZIPneo, H-RasF, H-RasGG) were treated with vehicle or FTI-277, membrane and cytosolic fractions were isolated and immunoprecipitated with anti-Raf antibody, and the resulting immunoprecipitates were blotted with anti-Ras antibody as described under "Experimental Procedures." Fig. 3 shows that Raf did not associate with Ras in pZIPneo cells which do not contain GTP-locked Ras. In contrast, H-RasF and H-RasGG cells contain Ras-Raf complexes in the membrane but not in the cytosolic fractions of untreated cells (Fig. 3). Treatment with FTI-277 resulted in the accumulation of Ras-Raf complexes in the cytoplasmic but not membrane fractions of H-RasF cells (Fig. 3). The lack of Ras-Raf interaction at the cell membrane and accumulation of these complexes in the cytoplasm occurred only in Ras-F but not Ras-GG cells in agreement with the Ras processing selectivity results of Fig. 2. Thus, our results demonstrate that inhibition with FTI-277 results in the accumulation of non-farnesylated cytosolic Ras that is capable of binding to Raf. The fact that non-processed Ras can associate with Raf in a non-membranous, cytoplasmic environment was confirmed by transfecting NIH 3T3 cells with a GTP-locked Ras that lacks a farnesylation site (Ras mutant with a leucine 61 oncogenic mutation and a serine 186 mutation (34)) and, therefore, remains in the cytoplasm. These cells were shown to contain only cytoplasmic Ras-Raf complexes when immunoprecipitated with Raf and blotted with anti-Ras antibodies (Fig. 3, *S186*). Thus, farnesylation is not required for Ras to bind to Raf. Furthermore, the fact that non-farnesylated Ras binds Raf in the cytoplasm gives support to an earlier suggestion that unprocessed GTP-locked Ras is a dominant negative form of Ras (35).

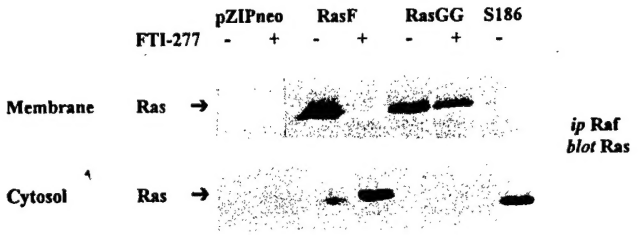

FIG. 3. **Effects of FTI-277 on Ras/Raf association.** pZIPneo, H-RasF, H-RasGG, and S186 cells were treated with vehicle or FTI-277 (10 μM), homogenized, and the membrane and cytosolic fractions were separated and immunoprecipitated by an anti-Raf antibody as described under "Experimental Procedures." The immunoprecipitates were then resolved by SDS-PAGE and immunoblotted with anti-Ras antibody. Data are representative of three independent experiments.

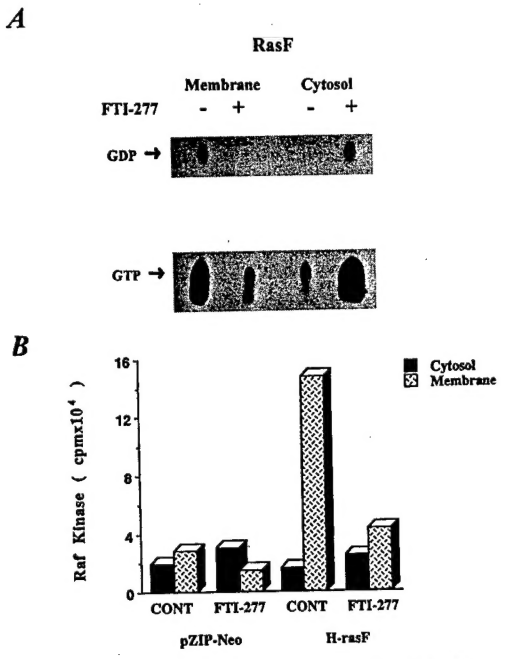

FIG. 4. **Effects of FTI-277 on Ras nucleotide binding and Raf kinase activity.** *A*, H-RasF cells were treated with vehicle or FTI-277, lysed, and the lysates were immunoprecipitated with anti-Ras antibody. GTP and GDP were then released from Ras and separated by TLC as described under "Experimental Procedures." *B*, pZIPneo and H-RasF cells were treated with vehicle or FTI-277, lysed, and cell lysates were immunoprecipitated with an anti-Raf antibody. Raf kinase was assayed as described under "Experimental Procedures." Data are representative of three independent experiments.

Since Raf binds Ras-GTP with much higher affinity than Ras-GDP (1–3), we determined the nucleotide state of Ras in the cytoplasmic Ras-Raf complexes as described under "Experimental Procedures." Fig. 4*A* shows that in H-RasF cells only membrane fractions contained GTP-locked Ras. Upon treatment with FTI-277, however, GTP-locked H-Ras was found primarily in the cytosol (Fig. 4*A*). Thus, the cytoplasmic form of H-Ras(61L) is still GTP-bound and can, therefore, still interact with Raf. We next determined the Ser/Thr kinase activity of Raf in Ras/Raf complexes by immunoprecipitating Raf and assaying for its ability to phosphorylate a 19-mer autophosphorylated peptide. Fig. 4*B* shows that oncogenic H-RasF induced activation of Raf at the plasma membrane and that treatment with FTI-277 suppressed this activation. More importantly, the cytoplasmic Ras/Raf complexes (Fig. 3) had basal levels of Raf kinase activity that were comparable with those of the parental NIH 3T3 cell line pZIPneo (Fig. 4*B*). Taken together, Figs. 3 and 4 demonstrate that oncogenic transformation with GTP-locked H-Ras results in the constitutive recruitment of Raf to

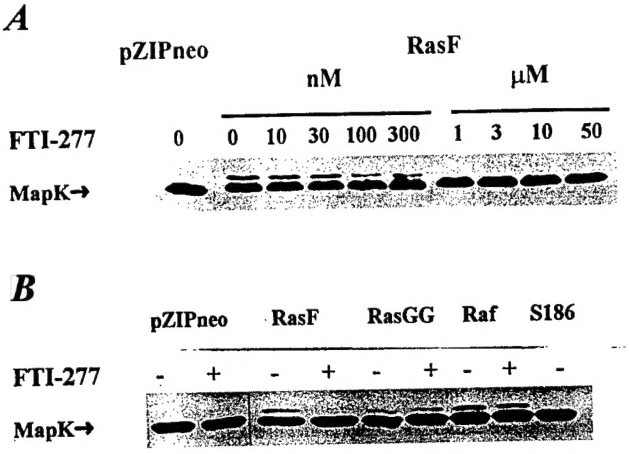

FIG. 5. **Effect of FTI-277 on oncogenic activation of MAPK.** *A*, H-RasF cells were treated with various concentrations of FTI-277, cells lysed, and lysates run on SDS-PAGE and immunoblotted with anti-MAPK antibody as described under "Experimental Procedures." *B*, pZIPneo, H-RasF, H-RasGG, Raf, and S186 cells were treated with vehicle or FTI-277 (10 μM), lysed, and cells lysates processed as for *A*. Data are representative of two independent experiments.

the plasma membrane and its subsequent activation. Furthermore, FTase inhibition by FTI-277 suppresses this activation by inducing the accumulation of Ras-Raf complexes in the cytoplasm where Ras is GTP-bound, but Raf kinase is not activated. The fact that Raf kinase is not activated when bound to Ras in a non-membranous environment is consistent with recent reports that indicate that Raf activation requires an as yet unidentified activating factor at the plasma membrane (36).

We then investigated the effects of FTI-277 on oncogenic Ras activation of MAPK, a Raf downstream signaling event (1–3). Oncogenic activation of MAPK can be easily detected, since the phosphorylated activated MAPK migrates slower in SDS-PAGE (29). Fig. 5*A* shows that NIH 3T3 cells transfected with pZIPneo contain only inactive MAPK but that upon transformation with oncogenic H-Ras, MAPK is activated (Fig. 5*A*). Pretreatment with FTI-277 results in a concentration-dependent inhibition of the constitutive activation of MAPK by oncogenic H-Ras. Concentrations as low as 300 nM were effective, and the inhibition was complete at 1 μM. Taken together, Figs. 2 and 5 demonstrate that at least 50% but less than 100% inhibition of H-Ras processing is required for inhibition of MAPK activation. To determine whether the inhibition of MAPK activation is due to selectively antagonizing H-Ras function we have used a series of NIH 3T3 cells lines transformed with various oncogenes. Fig. 5*B* shows that FTI-277 was able to block H-RasF but not H-RasGG activation of MAPK, and this is consistent with its ability to inhibit H-RasF but not H-RasGG processing. Selectivity of FTI-277 toward inhibition of Ras-dependent activation of MAPK was substantiated by using NIH 3T3 cells, where MAPK is constitutively activated by transformation with the Raf oncogene (33). Fig. 5*B* shows that oncogenic Raf activation of MAPK is not blocked by FTI-277, even though processing of endogenous Ras was inhibited in these cells (Fig. 2*B*). Taken together these results clearly demonstrate that FTI-277 is highly effective and selective in disrupting constitutive H-Ras-specific activation of MAPK.

Since K-Ras4B, the predominant form of Ras mutated in human tumors, is a much more efficient substrate (*CAAX* = CVIM) for FTase than is H-Ras (*CAAX* = CVLS) (13, 37), its processing has been difficult to disrupt. To determine whether or not FTI-277 inhibits K-Ras processing, we have treated K-Ras4B cells (NIH 3T3 cells transformed with oncogenic (valine 12) K-Ras4B-CVIM (17)) with FTI-277 (0–30 μM). Fig. 6

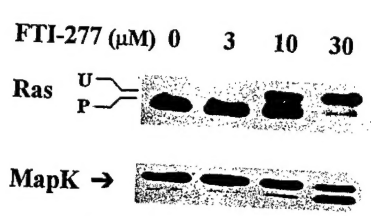

FIG. 6. **FTI-277 inhibits oncogenic K-Ras4B processing and activation of MAPK.** NIH 3T3 cells that overexpress oncogenic K-Ras4B were treated with FTI-277 (0, 3, 10, and 30 μM), and the cell lysates were immunoblotted with anti-Ras or anti-MAPK antibody as described under "Experimental Procedures." Data are representative of three and two independent experiments, respectively.

shows that FTI-277 inhibited K-Ras4B processing with an IC_{50} of 10 μM. Thus, inhibiting K-Ras4B processing (Fig. 6) requires 100-fold higher concentration than that needed for inhibition of H-Ras processing (Fig. 2*A*). This lower sensitivity to FTI-277 could be because K-Ras4B-CVIM is a much better substrate than H-Ras-CVLS (13, 37). An alternative explanation is that K-Ras4B-CVIM could be geranylgeranylated (37), especially when cellular FTase is inhibited. The fact that inhibition of K-Ras4B processing occurs only at concentrations that inhibit the processing of the geranylgeranylated Rap1A (Fig. 2*A*) is consistent with this latter possibility. We next determined whether the inhibition of K-Ras processing results in disruption of oncogenic K-Ras4B constitutive activation of MAPK. The same cell lysates that were blotted with anti-Ras antibody (Fig. 6) were reblotted with anti-MAPK antibody as described under "Experimental Procedures." Fig. 6 shows that NIH 3T3 cells that overexpress oncogenic K-Ras4B (17) contain mainly hyperphosphorylated (activated) MAPK. Treatment of these cells with FTI-277 (30 μM) inhibited oncogenic K-Ras4B constitutive activation of MAPK (Fig. 6). Furthermore, consistent with inhibition of Ras processing data (Fig. 6), higher concentrations were required to inhibit MAPK activation by K-Ras4B as compared with H-Ras. Nevertheless, the data clearly demonstrate for the first time that an FTase inhibitor disrupts both H- and K-Ras processing and oncogenic signaling.

Thus, we have designed an extremely potent and highly selective FTase inhibitor. FTI-277 inhibited H-Ras processing with concentrations as low as 10 nM, and processing was blocked by more than 95% at 3 μM. The most potent inhibitors previously reported blocked H-Ras processing completely only at 100 μM (28–30). The tremendous increase of potency in intact cells is due to increased hydrophobicity of the central portion of the peptidomimetic. FTI-277 inhibition of FTase resulted in the accumulation of non-farnesylated, GTP-locked H-Ras in the cytoplasm, where it was capable of binding Raf. This sequestration of Raf in the cytoplasm prevented its recruitment to the plasma membrane and subsequent activation. Thus, non-farnesylated cytoplasmic H-Ras could act as a dominant inhibitor by sequestering its downstream effector. Furthermore, FTI-277 was very selective in antagonizing H-Ras-specific signaling. The fact that FTI-277 suppressed only H-RasF but not H-RasGG or Raf oncogenic signaling demonstrates that the suppression is due to inhibition of H-Ras function and not the function of other farnesylated proteins that may be required for H-Ras transformation. Finally, we demonstrated for the first time that an FTase inhibitor can inhibit K-Ras processing and signaling but at much higher doses than required for H-Ras. Furthermore, we have recently demonstrated that FTI-276 and FTI-277 inhibit tumor growth in nude mice of a human lung carcinoma that has a K-Ras mutation and a p53 deletion (38). Since the great majority of human tumors with Ras mutations are of the K-type rather than the H-type, this finding is critical to further development of these agents as anti-cancer drugs.

Note Added in Proof—We have recently demonstrated that oncogenic K-Ras4B processing and signaling are inhibited potently with a GGTase I-specific inhibitor (39).

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